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Plants with reduced activity of a Class 3 branching enzyme

Description

The present invention relates to plant cells and plants, which are genetically modified, wherein the genetic modification leads to the reduction of the activity of a Class 3 vegetable branching enzyme in comparison with corresponding wild type plant cells or wild type plants that have not been genetically modified. Furthermore, the present invention relates to means and methods for the manufacture of such plant cells and plants. Plant cells and plants of this type synthesise a modified starch. The present invention therefore also relates to the starch synthesised by the plant cells and plants according to the invention as well as to methods for the manufacture of the starch and to the manufacture of starch derivatives of this starch. Furthermore, the present invention relates to nucleic acids coding a Class 3 branching enzyme, vectors, host cells, plant cells and plants containing such nucleic acid molecules.

With regard to the increasing importance currently attributed to vegetable constituents as renewable raw material sources, one of the tasks of biotechnological research is to endeavour to adapt these vegetable raw materials to suit the requirements of the processing industry. Furthermore, in order to enable regenerating raw materials to be used in as many areas of application as possible, it is necessary to achieve a large variety of materials.

Polysaccharide starch is made up of chemically uniform base components, the glucose molecules, but constitutes a complex mixture of different molecule forms, which exhibit differences with regard to the degree of polymerisation and branching, and therefore differ strongly from one another in their physical-chemical characteristics. Discrimination is made between amylose starch, an essentially unbranched polymer made from α-1,4-glycosidically linked glucose units, and the

amylopectin starch, a branched polymer, in which the branches come about by the occurrence of additional α-1,6-glycosidic links. A further essential difference between amylose and amylopectin lies in the molecular weight. While amylose, depending on the origin of the starch, has a molecular weight of $5x10^5$ – 10^6 Da, that of the amylopectin lies between 10^7 and 10^8 Da. The two macromolecules can be differentiated by their molecular weight and their different physical-chemical characteristics, which can most easily be made visible by their different iodine bonding characteristics.

Amylose has long been looked upon as a linear polymer, consisting of α-1,4-glycosidically linked α-D-glucose monomers. In more recent studies, however, the presence of α-1,6-glycosidic branching points (ca. 0.1%) has been shown (Hizukuri and Takagi, Carbohydr. Res. 134, (1984), 1-10; Takeda et al., Carbohydr. Res. 132, (1984), 83-92).

Amylopectin constitutes a complex mixture of differently branched glucose chains. In contrast to amylose, amylopectin is more strongly branched. According to textbook information (Voet and Voet, Biochemistry, John Wiley & Sons, 1990), on average, the α-1,6 branches occur every 24 to 30 glucose residues. This is equivalent to a degree of branching of ca. 3% - 4%. The figures for the degree of branching are variable and are dependent on the origin (e.g. plant species, plant type etc.) of the appropriate starch. In typical plants used for the industrial production of starch, such as maize, wheat or potato, for example, the synthesised starch consists of ca. 20% - 30% amylose starch and ca. 70% - 80% amylopectin starch.

The functional characteristics of the starch, along with the amylose/amylopectin ratio ~~and the phosphate~~ content, are strongly affected by the molecular weight, the pattern of the side chain distribution, the ion concentration, the lipid and protein content, the average grain size of the starch and the grain morphology of the starch etc. At the same time, by way of example, the solubility, the retrogradation behaviour, ~~the water bonding capability~~, the film formation characteristics, the viscosity, the sticking ~~characteristics, the freezing-thawing stability~~, the acid stability, the gelling strength

etc. must be mentioned as important functional characteristics. The grain size of the starch can also be important for different applications.

Branching enzymes, which are also abbreviated by the designation "BE" (from Branching Enzyme; E.C. 2.4.1.18), catalyse the introduction of α-1,6 branches in α-1,4-glucans. Branching enzymes and the nucleic or amino acid sequences that characterise them are known from widely different organisms, such as bacteria, microbial fungi, mammals, algae and higher plants, for example. As only plants synthesise starch, while the above-mentioned non-vegetable organisms (e.g. bacteria, fungi and mammals) synthesise glycogen, the related branching enzymes, which are involved in the synthesis of the appropriate polymer, can also be sub-divided into glycogen branching enzymes and starch branching enzymes. Plants are therefore starch branching enzymes, which are often also referred to as Q-enzymes in older literature.

In all plant species that have been investigated up to now, the branching enzymes described can be associated with two different classes (Burton et al., 1995, Plant Journal 7, 3-15; Mizuno et al., 2001, Plant Cell Physiol. 42(4), 349-357). The association with these classes, sometimes designated in the literature with A or 2 respectively and B or 1 respectively, is based on the comparison of derived protein sequences.

As different nomenclatures have been used in the past for designating and classifying branching enzymes, Smith-White and Preiss (1994, Plant Molecular Biology Reporter 12, 67-71) (1994, Plant Molecular Biology Reporter 12, 67-71) have proposed a system for standardising this nomenclature, in which the association with the two classes of vegetable branching enzymes is also based on the comparison of derived protein sequences (Larsson et al., 1998, Plant Mol. Biol. 37, 505-511). According to this nomenclature, those vegetable branching enzymes, the amino acid sequence of which has a higher degree of identity with that of branching enzyme I of maize (GenBank Acc: D11081), is to be designated as a Class 1 branching enzyme, and those vegetable branching enzymes, the coding amino acid sequence of which has a higher degree of identity with that of branching enzyme II of maize (GenBank

Acc: AF072725), is to be designated as a Class 2 branching enzyme. The designation of gene products, which are coding for branching enzymes, are, in accordance with the nomenclature of Smith-White and Preiss, to be incorporated in the already existing nomenclature by means of E.C. numbers. This results in the so-called GPN (Gene Product Number) Codes for the two classes, namely GPN 2.2.4.1.18:1 for Class 1 branching enzymes and GPN 2.2.4.18:2 for Class 2 branching enzymes.

The following vegetable or starch branching enzymes therefore belong to Class 1 (GPN 2.2.1.18:1) according to the nomenclature proposed by Smith-White and Preiss (1994, Plant Molecular Biology Reporter 12, 67-71):
BE I from *Aegilops tauschii* (GenBank Acc: AF525746), BE I from barley (GenBank Acc: AY304541), BE from tapioca (GenBank Acc: X77012), BE I (frequently also described as BE 1) from rice (GenBank Acc: D11082, D10752, D10838), BE 3 from bean (GenBank Acc: AB029549), BE II from pea (GenBank Acc: X80010), BE from millet (GenBank Acc: AF169833), BE I from potato (GenBank Acc: Y08786, X69805), BE from wheat (GenBank Acc: Y12320, AF076679, AF002820) and BE I from maize (GenBank Acc: D11081, AAO20100, E03435, AY176762, U17897, AF072724).
At the same time, the amino acid sequences for different Class 1 branching enzymes each have an identity of more than 60% with the amino acid sequence of branching enzyme I from maize (GenBank Acc: D11081).

Branching enzymes, which belong to Class 2 (GPN 2.2.1.18:2) according to the nomenclature proposed by Smith-White and Preiss (1994, Plant Molecular Biology Reporter 12, 67-71) are, for example, BE IIa from *Aegilops tauschii* (GenBank Acc: AF338431, WO 9914314), BE2-1 and BE2-2 from *Arabidosis thaliana* (BE2-1 GenBank Acc: NM_129196 CAA04134; BE2-2 GenBank Acc: CAB82930, NM_120446), BE IIa and BE IIb from barley (BE IIa GenBank Acc: AF064560; BE IIb GenBank Acc: AF064561), BE II from sweet potato (GenBank Acc: AB071286), BE III and BE IV (frequently also described as BE 3 or BE 4 respectively) from rice (BE iii GenBank Acc: D16201; BE IV GenBank Acc: AB023498), BE 1 from bean (GenBank Acc: AB029548), BE I from pea (GenBank Acc: X80009), BE IIb from millet (GenBank Acc: AY304540), BE II from potato (GenBank Acc: AJ000004,

AJ011885, AJ011888, AJ011889, AJ011890), BE II or BE IIa from wheat (GenBank Acc: Y11282, AF286319, AF338432, U66376) and BE II, or BE IIb from maize (BE II GenBank Acc: AAA18571, T02981; BE IIb GenBank Acc: AF072725, L08065). At the same time, the amino acid sequences for different Class 2 branching enzymes each have an identity of more than 60% with the amino acid sequence of branching enzyme IIb from maize (GenBank Acc: AF072725).

Vegetable or starch branching enzymes belong to the family of alpha-amylolytic enzymes (Svensson, 1994, Plant Molecular Biology 25, 141-157; Jespersen et al., 1991, Biochem J. 280, 51-55) and, with regard to the amino acid sequence, have four conserved domains (Baba et al., 1991, Biochem. Biophys. Res. Commun. 181(1), 87-94; Kuriki et al., 1996, J. of Protein Chemistry 15(3), 305-313).

Structural predictions based on mathematical calculations derived from experimental data such as protein crystal structures (Pfam: http://hits.isb-sib.ch/cgi-bin/PFSCAN?) show that all previously known branching enzymes from higher plants have two domains: an alpha-amylase domain and an iso-amylase domain. Here, the iso-amylase domain lies closer to the N-terminus of the protein than the alpha-amylase domain.

Plants are known, for example, which have a reduced activity of a Class 2 branching enzyme due to a mutation. These include the so-called "*amylose extender*" (*ae*) mutants from maize (Stindard et al., 1993, Plant Cell 5, 1555-1566; Boyer and Preiss, 1978, Biochem. Biophys. Res. Commun. 80, 169-175) and rice (Mizuno et al., 1993, J. Biol. Chem. 268, 19084-19091), as well as the "*rugosus*" (*r*) mutation in pea (Smith, 1988, Planta 175, 270-279; Bhattacharyya et al., 1990, Cell 60, 115-122). All these mutants are distinguished by the fact that they synthesise a starch, which has an increased amylose content in comparison with starches from corresponding plants, which do not have this mutation.

Furthermore, genetically modified potato plants are described, in which the activity of a BE I (Class 1) branching enzyme (Kossmann et al., 1991, Mol Gen Genet 230, 39-44; Safford et al., 1998, Carbohydrate Polymers 35, 155-168), or the activity of a BEII

(Class 2) branching enzyme (Jobling et al., 1999, The Plant Journal 18), or the activity of a BEI and BEII branching enzyme (Schwall et al., 2000, Nature Biotechnology 18, 551- 554, Jobling et al., 2003, Nature Biotechnology 21, 77-80) are reduced.

Previously, it has been possible to associate all vegetable branching enzymes to one or both of the classes described above. Plant cells or plants, which have a reduced activity of a branching enzyme, which cannot be associated with these classes, are unknown.

The object of the present invention is therefore based on providing modified starches, new plant cells and/or plants, which synthesise such a modified starch, as well as means and methods for producing said plants.

This problem is solved by the embodiments described in the claims.

The present invention therefore relates to genetically modified plant cells and genetically modified plants, characterised in that the plant cells or plants have a reduced activity of at least one Class 3 branching enzyme in comparison with corresponding wild type plant cells or wild type plants that have not been genetically modified.

A first aspect of the present invention relates to a plant cell or plant, which is genetically modified, wherein the genetic modification leads to the reduction of the activity of at least one Class 3 branching enzyme in comparison with corresponding wild type plant cells or wild type plants that have not been genetically modified.

At the same time, the genetic modification can be any genetic modification, which leads to a reduction of the activity of at least one Class 3 branching enzyme in comparison with corresponding wild type plant cells or wild type plants that have not been genetically modified.

In conjunction with the present invention, the term "wild type plant cell" means that the plant cells concerned were used as starting material for the manufacture of the plant cells according to the invention, i.e. their genetic information, apart from the introduced genetic modification, corresponds to that of a plant cell according to the invention.

In conjunction with the present invention, the term "wild type plant" means that the plants concerned were used as starting material for the manufacture of the plants according to the invention, i.e. their genetic information, apart from the introduced genetic modification, corresponds to that of a plant according to the invention.

In conjunction with the present invention, the term "corresponding" means that, in the comparison of several objects, the objects concerned that are compared with one another have been kept under the same conditions. In conjunction with the present invention, the term "corresponding" in conjunction with wild type plant cell or wild type plant means that the plant cells or plants, which are compared with one another, have been raised under the same cultivation conditions and that they have the same (cultivation) age.

In an embodiment of the present invention, the genetic modification of the plant cells according to the invention or the plants according to the invention is brought about by mutagenesis of one or more genes. The type of mutation is not important, as long as it leads to a reduction in the activity of a Class 3 branching enzyme.

In conjunction with the present invention, the term "mutagenesis" is to be understood to mean any type of introduced mutation, such as deletions, point mutations (nucleotide exchanges), insertions, inversions, gene conversions or chromosome translocations, for example.

Here, the mutation, which leads to the reduction of the activity of at least one endogenous Class 3 branching enzyme, can be produced by the use of chemical agencies or energy-rich radiation (e.g. x-rays, neutron radiation, gamma radiation or UV radiation).

Agencies, which can be used to produce chemically induced mutations, and the mutations resulting from the effect of the corresponding mutagens are, for example described in Ehrenberg and Husain, 1981, (Mutation Research 86, 1-113), Müller, 1972 (Biologisches Zentralblatt 91 (1), 31-48). The production of rice mutants using gamma radiation, ethyl methane sulphonate (EMS), N-methyl-N-nitrosurea or sodium azide (NaN_3) is described, for example, in Jauhar and Siddiq (1999, Indian Journal of Genetics, 59 (1), 23-28), in Rao (1977, Cytologica 42, 443-450), Gupta and Sharma (1990, Oryza 27, 217-219) and Satoh and Omura (1981, Japanese Journal of Breeding 31 (3), 316-326). The production of wheat mutants using NaN_3 or maleic hydrazide is described in Arora et al. (1992, Annals of Biology 8 (1), 65-69). An overview of the production of wheat mutants using different types of energy-rich radiation and chemical agencies is presented in Scarascia-Mugnozza et al. (1993, Mutation Breeding Review 10, 1-28). Svec et al. (1998, Cereal Research Communications 26 (4), 391-396) describes the use of N-ethyl-N-nitrosurea for producing mutants in triticals. The use of MMS (methyl methane sulphonic acid) and gamma radiation for the production of millet mutants is described in Shashidhara et al. (1990, Journal of Maharashtra Agricultural Universities 15 (1), 20-23).

The manufacture of mutants in plant species, which mainly propagate vegetatively, has been described, for example, for potatoes, which produce a modified starch (Hovenkamp-Hermelink et al. (1987, Theoretical and Applied Genetics 75, 217-221) and for mint with increased oil yield or modified oil quality (Dwivedi et al., 2000, Journal of Medicinal and Aromatic Plant Sciences 22, 460-463).
All these methods are basically suitable for manufacturing the plant cells according to the invention and the plants according to the invention.

Mutations in the appropriate genes, in particular in genes coding a Class 3 branching enzyme, can be found with the help of methods known to the person skilled in the art. In particular, analyses based on hybridisations with probes (Southern Blot), amplification by means of polymerase chain reaction (PCR), sequencing of related genomic sequences and the search for individual nucleotide exchanges can be used for this purpose. A method of identifying mutations based on hybridisation patterns is, for example, the search for restriction fragment length differences (Restriction

Fragment Length Polymorphism, RFLP) (Nam et al., 1989, The Plant Cell 1, 699-705; Leister and Dean, 1993, The Plant Journal 4 (4), 745-750). A method based on PCR is, for example, the analysis of amplified fragment length differences (Amplified Fragment Length Polymorphism, AFLP) (Castiglioni et al., 1998, Genetics 149, 2039-2056; Meksem et al., 2001, Molecular Genetics and Genomics 265, 207-214; Meyer et al., 1998, Molecular and General Genetics 259, 150-160). The use of amplified fragments cut with restriction endonucleases (Cleaved Amplified Polymorphic Sequences, CAPS) can be called upon for the identification of mutations (Konieczny and Ausubel, 1993, The Plant Journal 4, 403-410; Jarvis et al., 1994, Plant Molecular Biology 24, 685-687; Bachem et al., 1996, The Plant Journal 9 (5), 745-753). Methods for the determination of SNPs have been described by Qi et al. (2001, Nucleic Acids Research 29 (22), e116) Drenkard et al. (2000, Plant Physiology 124, 1483-1492) and Cho et al. (1999, Nature Genetics 23, 203-207) amongst others. Methods, which allow several plants to be investigated for mutations in certain genes in a short time, are particularly suitable. Such a method, so-called TILLING (Targeting Induced Local Lesions IN Genomes), has been described by McCallum et al. (2000, Plant Physiology 123, 439-442).

These methods are basically suitable for identifying plant cells according to the invention and plants according to the invention.

Hoogkamp et al. (2000, Potato Research 43, 179-189) have manufactured stable monoploid mutants starting from a potato mutant (*amf*), which was manufactured by means of chemical mutagens. These plants do not synthesise any more active enzyme for a starch synthesis connected to the starch grain (GBSS I) and therefore produce an amylose-free starch. The monoploid potato plants obtained can be used as starting material for further mutageneses in order to identify plants, which synthesise a starch with modified characteristics. The plant cells according to the invention and plants according to the invention, which produce a starch according to the invention, can be identified and isolated by appropriate methods.

The plant cells according to the invention and the plants according to the invention have a reduction of the activity of at least one Class 3 branching enzyme in

comparison with corresponding wild type plant cells that have not been genetically modified.

Here, within the framework of the present invention, the term "reduction of activity" means a reduction in the expression of endogenous genes, which code Class 3 branching enzymes, and/or a reduction in the quantity of protein of a Class 3 branching enzyme in the plant cells and/or a reduction in the enzymatic activity of Class 3 branching enzymes in the plant cells.

The reduction in the expression can, for example, be determined by measuring the quantity of transcripts coding Class 3 branching enzyme, e.g. using Northern blot analysis or RT-PCR. Here, a reduction preferably means a reduction in the amount of transcripts in comparison with corresponding plant cells that have not been genetically modified by at least 50%, in particular by at least 70%, preferably by at least 85% and particularly preferably by at least 95%.

The reduction in the amount of protein of a Class 3 branching enzyme, which results in a reduced activity of this protein in the plant cells concerned, can, for example, be determined by immunological methods such as Western blot analysis, ELISA (Enzyme Linked Immuno Sorbent Assay) or RIA (Radio Immune Assay). Here, a reduction preferably means a reduction in the amount of Class 3 branching enzyme protein in comparison with corresponding plant cells that have not been genetically modified by at least 50%, in particular by at least 70%, preferably by at least 85% and particularly preferably by at least 95%.

Within the framework of the present invention, the term "branching enzyme" (α-1,4-glucan: α-1,4- glucan 6-glycosyltransferase, E.C. 2.4.1.18) is understood to mean a protein, which catalyses a transglycosylation reaction, in which α-1,4 links of an α-1,4-glucan donor are hydrolysed and the thereby released α-1,4-glucan chains are transferred to an α-1,4-glucan acceptor chain and, in doing so, are transformed into α-1,6-links. In particular, within the framework of the present invention, the term

"branching enzyme" is to be understood to mean a vegetable branching enzyme, i.e. a starch branching enzyme.

The activity of a branching enzyme can be demonstrated, for example, with the help of native acrylamide gel electrophoresis. In doing so, proteins are first separated electrophoretically and, after incubation in buffers containing an activity, which synthesises linear α-1,4-glucan chains (e.g. starch phosphorylase a) and its substrate (e.g. glucose-6-phosphate), the corresponding gels are coloured with iodine (Kimihiko et al., 1980, Analytical Biochemistry 108, 16-24).
Furthermore, branching enzymes in microbial organisms, such as the *E. coli* strain KV832 for example (Kiel et al., 1987 Mol. Gen. Genet 207: 294-301), which do not synthesise branched α-glucans, can be expressed. If an activity of a branching enzyme is introduced into the microbial organism due to the expression of a foreign gene in such strains (e.g. *E. coli* KV832), then the branching enzyme activity can be demonstrated by treating colonies of these organisms with iodine vapour, for example. Colonies, which synthesise linear α-1,4-glucans, turn blue in this test, while colonies, which synthesise branched glucans by expressing an additional enzymatic activity of a branching enzyme, turn reddish-brown after treating with iodine vapour. It is also possible to express proteins in phosphoglucomutase mutants of *E. coli* to identify a branching enzyme activity of appropriate proteins (Buettcher et al., 1999, Biochem. Biophys. Acta 1432, 406-412).
A further possibility of demonstrating branching enzyme activity of proteins is the use of a reaction stimulated by phosphorylase a and the subsequent separation of the products by means of thin film chromatography (Almstrupp et al., 2000, Analytical Biochemistry 286, 297-300).
Branching enzyme activities can also be demonstrated with the help of the methods described in Guan and Preiss (1993, Plant Physiol. 102. 1269- 1273) and Kuriki et al. (1996, J. of Protein Chemistry 15, 305-313).

In conjunction with the present invention, the term "Class 3 branching enzyme" is to be understood as a branching enzyme, which has a higher degree of identity with the amino acid sequence shown in SEQ ID NO 4 than with that of the branching enzyme BE I from maize (GenBank Acc: D11081) or with that of the branching enzyme BE IIb

from maize (GenBank Acc: AF072725). Preferably, the Class 3 branching enzyme comes from starch-storing plants, particularly preferably from plant species of the genus *Solanum*, especially preferably from *Solanum tuberosum*.

In a further embodiment of the present invention, amino acid sequences coding Class 3 branching enzymes have an identity of at least 60% with the sequence shown in SEQ ID NO 4, in particular of at least 70%, preferably of at least 80% and particularly preferably of at least 90% and especially preferably of at least 95%.

According to the invention, Class 3 branching enzymes have an iso-amylase domain (Pfam acc.: Pf02922) and an alpha-amylase domain (Pfam acc: Pf00128). According to the invention, the iso-amylase domain and the alpha-amylase domain in amino acid sequences coding branching enzymes are separated from one another by the presence of further amino acids, which do not belong to these two domains.
Class 3 branching enzymes according to the invention are distinguished by the fact that the iso-amylase domain is separated from the alpha-amylase domain by a greater number of amino acids than the iso-amylase domain and the alpha-amylase domain of Class 1 and 2 branching enzymes.
Class 3 branching enzymes according to the invention are preferably distinguished with regard to their amino acid sequence by the fact that they have at least 70, preferably at least 100, particularly preferably at least 130 and especially preferably at least 198 amino acids between the iso-amylase domain and the alpha-amylase domain. In a further embodiment of the present invention, in the case of an amino acid sequence coding a Class 3 branching enzyme, the C-terminal end of the iso-amylase domain is separated from the N-terminal beginning of the alpha-amylase domain by 70 to 198, preferably by 100 to 198, particularly preferably by 130 to 198 and especially particularly preferably by 150 to 198 amino acids.

With the help of the Pfam database (Batemann et al., 2002, Nucleic Acids Research 30, 276-280; accessible via http://www.sanger.ac.uk/Software/Pfam/, http://www.cgb.ki.se/Pfam/; http://pfam.jouy.inra.fr/ or http://pfam.wustl.edu/), it is possible for the person skilled in the art to determine whether amino acid sequences

already have known domains (e.g. an iso-amylase domain and/or an alpha-amylase domain).

Pfam is a database put together by experts, which classifies amino acid sequences into so-called families. Here, the assignment of an amino acid sequence to a family is carried out on the basis of so-called domains, which are to be looked upon as functional and structural components of proteins. A domain is defined as a structural unit or a repeatedly occurring amino acid sequence unit, which can occur in proteins with widely different functions. Along with information relating to the amino acid sequence of known proteins, further knowledge (e.g. evidence of the enzymatic activity, crystal structure data) is also used for the assignment of a protein to a family. Each family is assigned a name and an "accession" number (e.g. Name: Isoamylase_N, acc: PF02922). A constituent part of each family in the Pfam database is, amongst other things, a so-called "seed alignment". The "seed alignment" contains the amino acid sequences of representative proteins of a family. Starting from "seed alignments", a so-called profile HMM ("profile Hidden Markov Model"; overview article in: Durbin et al., "Biological Sequence Analysis: Probabilistic Models of Proteins and Nucleic Acids", Cambridge University Press, 1998, ISBN 0-521-62041-4) is produced using the HMMER 2 software (freely available under http://hmmer.wustl.edu/). The HMMs produced have names and are stored in the Pfam database specifically for the correspondingly assigned domains. In contrast to classical, multiple "alignments" (e.g. produced using the Clustal W program or the Blossum62 algorithm), HMMs are based on a valid statistical theory (Bayes theory of conditional probability, Markoff chains) and enable an interrogation sequence (query) to be assigned to a family based on the use of position-specific evaluation matrices. This enables an assignment to be made even when there are considerable differences in the amino acid sequences between the interrogation sequence (query) and a comparison sequence (e.g. amino acid sequence entry in a database).

The domain structure of the amino acid sequence concerned can be determined by means of a comparison of the HMMs stored in the Pfam database with amino acid sequences, which are entered as a so-called interrogation sequence (query) (e.g. under http://hits.isb-sib.ch/cgi-bin/PFSCAN?).

In conjunction with the present invention, the term "iso-amylase domain" is to be understood to mean a Pfam iso-amylase domain (acc: Pf02922). At the same time, the HMM describing this Pfam iso-amylase domain is to be produced with the HMMER 2 [2.3.1] software, starting from a "seed alignment", which contains the amino acid sequences shown in Table 1. In conjunction with the present invention, the "seed alignment" is produced by means of the ClustalW program (Thompson et al., Nucleic Acids Research 22 (1994), 4673-4680; see below). The following settings must be chosen to produce the appropriate HMMs: Build Method of HMM: hmmbuild –F HMM_ls, hmmcalibrate –seed 0 HMM_ls; Gathering cutoff: 2.3 2.3; Trusted cutoff: 2.3 2.2; Noise cutoff: 2.1 2.1). Further information for producing the HMM of the Pfam iso-amylase domain (acc: Pf02922) is given in Table 3.

In conjunction with the present invention, the term "alpha-amylase domain" is to be understood to mean a Pfam alpha-amylase domain (acc: Pf00128). At the same time, the HMM describing this Pfam alpha-amylase domain is to be produced with the HMMER 2 [2.3.1] software, starting from a "seed alignment", which contains the amino acid sequences shown in Table 2. Here, the "seed alignment" is produced by means of HMM_simulated_annealing (http://www.psc.edu/general/software/packages/hmmer/manual/node11.html#SECTION00321000000000000000). The following settings must be chosen to produce the appropriate HMM: Build Method of HMM: hmmbuild –F HMM_ls, hmmcalibrate –seed 0 HMM_ls; Gathering cutoff: -82.0 –82.0; Trusted cutoff: -81.7 –81.7; Noise cutoff: -82.7 –82.7). Further information for producing the HMM of the Pfam alpha-amylase domain (acc: Pf00128) is given in Table 4.

In conjunction with the present invention, the term "Class 3 branching enzyme gene" is to be understood to mean a nucleic acid molecule (cDNA, DNA), which codes a Class 3 branching enzyme, preferably a Class 3 branching enzyme from starch-storing plants, particularly preferably from plant species of the genus *Solanum*, especially preferably from *Solanum tuberosum*.

A preferred embodiment of the present invention relates to a genetically modified plant cell according to the invention or a genetically modified plant according to the

invention, wherein the genetic modification consists in the introduction of at least one foreign nucleic acid molecule into the genome of the plant cell or into the genome of the plant.

In this context, the term "genetic modification" means the introduction of homologous and/or heterologous foreign nucleic acid molecules into the genome of a plant cell or into the genome of a plant, wherein said introduction of these molecules leads to a reduction of the activity of a Class 3 branching enzyme.

The plant cells according to the invention or plants according to the invention are modified with regard to their genetic information by the introduction of a foreign nucleic acid molecule. The presence or the expression of the foreign nucleic acid molecule leads to a phenotypic change. Here, "phenotypic" change means preferably a measurable change of one or more functions of the cells. For example, the genetically modified plant cells according to the invention and the genetically modified plants according to the invention exhibit a reduction of the activity of a Class 3 branching enzyme due to the presence or on the expression of the introduced nucleic acid molecule.

In conjunction with the present invention, the term "foreign nucleic acid molecule" is understood to mean such a molecule that either does not occur naturally in the corresponding wild type plant cells that have not been genetically modified, or that does not occur naturally in the concrete spatial arrangement in wild type plant cells that have not been genetically modified, or that is localised at a place in the genome of the wild type plant cell at which it does not occur naturally. Preferably, the foreign nucleic acid molecule is a recombinant molecule, which consists of different elements, the combination or specific spatial arrangement of which does not occur naturally in vegetable cells.

In principle, the foreign nucleic acid molecule can be any nucleic acid molecule, which effects a reduction of the activity of a Class 3 branching enzyme in the plant cell or plant.

In conjunction with the present invention, the term "genome" is to be understood to mean the totality of the genetic material present in a vegetable cell. It is known to the

person skilled in the art that, as well as the cell nucleus, other compartments (e.g. plastids, mitochondrions) also contain genetic material.

In a further embodiment, the plant cells according to the invention and the plants according to the invention are characterised in that the foreign nucleic acid molecule codes a Class 3 branching enzyme, preferably a Class 3 branching enzyme from starch-storing plants, particularly preferably from plants of a species of the genus *Solanum*, especially preferably from *Solanum tuberosum*.

In a particularly preferred embodiment, the foreign nucleic acid molecule codes a Class 3 branching enzyme with the amino acid sequence specified in SEQ ID NO 4.

A large number of techniques are available for the introduction of DNA into a vegetable host cell. These techniques include the transformation of vegetable cells with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as the transformation medium, the fusion of protoplasts, injection, the electroporation of DNA, the introduction of DNA by means of the biolistic approach as well as other possibilities.

The use of agrobacteria-mediated transformation of plant cells has been intensively investigated and adequately described in EP 120516; Hoekema, IN: The Binary Plant Vector System Offsetdrukkerij Kanters B.V., Alblasserdam (1985), Chapter V; Fraley et al., Crit. Rev. Plant Sci. 4, 1-46 and by An et al. EMBO J. 4, (1985), 277-287. For the transformation of potato, see Rocha-Sosa et al., EMBO J. 8, (1989), 29-33, for example.

The transformation of monocotyledonous plants by means of vectors based on agrobacterium transformation has also been described (Chan et al., Plant Mol. Biol. 22, (1993), 491-506; Hiei et al., Plant J. 6, (1994) 271-282; Deng et al, Science in China 33, (1990), 28-34; Wilmink et al., Plant Cell Reports 11, (1992), 76-80; May et al., Bio/Technology 13, (1995), 486-492; Conner and Domisse, Int. J. Plant Sci. 153 (1992), 550-555; Ritchie et al, Transgenic Res. 2, (1993), 252-265). An alternative system to the transformation of monocotyledonous plants is transformation by means of the biolistic approach (Wan and Lemaux, Plant Physiol. 104, (1994), 37-48; Vasil

et al., Bio/Technology 11 (1993), 1553-1558; Ritala et al., Plant Mol. Biol. 24, (1994), 317-325; Spencer et al., Theor. Appl. Genet. 79, (1990), 625-631), protoplast transformation, electroporation of partially permeabilised cells and the introduction of DNA by means of glass fibres. In particular, the transformation of maize has been described in the literature many times (cf. e.g. WO95/06128, EP0513849, EP0465875, EP0292435; Fromm et al., Biotechnology 8, (1990), 833-844; Gordon-Kamm et al., Plant Cell 2, (1990), 603-618; Koziel et al., Biotechnology 11 (1993), 194-200; Moroc et al., Theor. Appl. Genet. 80, (1990), 721-726).

The successful transformation of other types of cereal has also already been described, for example for barley (Wan and Lemaux, see above; Ritala et al., see above; Krens et al., Nature 296, (1982), 72-74) and for wheat (Nehra et al., Plant J. 5, (1994), 285-297). All the above methods are suitable within the framework of the present invention.

Amongst other things, the plant cells according to the invention and the plants according to the invention can be differentiated from wild type plant cells and wild type plants respectively in that they contain a foreign nucleic acid molecule, which does not occur naturally in wild type plant cells or wild type plants, or in that such a molecule is present integrated at a place in the genome of the plant cell according to the invention or in the genome of the plant according to the invention at which it does not occur in wild type plant cells or wild type plants, i.e. in a different genomic environment. Furthermore, plant cells according to the invention and plants according to the invention of this type differ from wild type plant cells and wild type plants respectively in that they contain at least one copy of the foreign nucleic acid molecule stably integrated within their genome, possibly in addition to naturally occurring copies of such a molecule in the wild type plant cells or wild type plants. If the foreign nucleic acid molecule(s) introduced into the plant cells according to the invention or into the plants according to the invention is (are) additional copies of molecules already occurring naturally in the wild type plant cells or wild type plants respectively, then the plant cells according to the invention and the plants according to the invention can be differentiated from wild type plant cells or wild type plants respectively in particular in that this additional copy or these additional copies is (are) localised at places in the genome at which it does not occur (or they do not occur) in

wild type plant cells or wild type plants. This can be verified, for example, with the help of a Southern blot analysis.

Furthermore, the plant cells according to the invention and the plants according to the invention can preferably be differentiated from wild type plant cells or wild type plants respectively by at least one of the following characteristics: If the foreign nucleic acid module that has been introduced is heterologous with respect to the plant cell or plant, then the plant cells according to the invention or plants according to the invention have transcripts of the introduced nucleic acid molecules. These can be verified, for example, by Northern blot analysis or by RT-PCR (Reverse Transcription Polymerase Chain Reaction). Plant cells according to the invention and plants according to the invention, which express an antisense and/or an RNAi transcript, can be verified, for example, with the help of specific nucleic acid probes, which are complimentary to the RNA (occurring naturally in the plant cell), which is coding for the protein.

If the foreign nucleic acid module that has been introduced is homologous with respect to the plant cell or plant, the plant cells according to the invention or plants according to the invention can be differentiated from wild type plant cells or wild type plants respectively due to the additional expression of the introduced foreign nucleic acid molecule, for example. The plant cells according to the invention and the plants according to the invention preferably contain (sense and/or antisense) transcripts of the foreign nucleic acid molecules. This can be demonstrated by Northern blot analysis, for example, or with the help of so-called quantitative PCR.

In a special embodiment, the plant cells according to the invention and the plants according to the invention are transgenic plant cells or transgenic plants respectively.

In a further embodiment, the present invention relates to plant cells according to the invention and plants according to the invention wherein the foreign nucleic acid molecule is chosen from the group consisting of

a) Nucleic acid molecules, which code a protein with the amino acid sequence given under Seq ID NO 4;

b) Nucleic acid molecules, which code a protein, the amino acid sequence of which has an identity of at least 50% with the amino acid sequence given under SEQ ID NO: 4;

c) Nucleic acid molecules, which include the nucleotide sequence shown under Seq ID NO 3 or a complimentary sequence;

d) Nucleic acid molecules, the nucleic acid sequence of which has an identity of at least 50% with the nucleic acid sequences described under a) or c);

e) Nucleic acid molecules, which hybridise with at least one strand of the nucleic acid molecules described under a) or c) under stringent conditions;

f) Nucleic acid molecules, the nucleotide sequence of which deviates from the sequence of the nucleic acid molecules identified under a), b), c), d), e) or f) due to the degeneration of the genetic code; and

g) Nucleic acid molecules, which represent fragments, allelic variants and/or derivatives of the nucleic acid molecules identified under a), b), c), d), e) or f).

A further embodiment of the present invention relates to plant cells according to the invention and plants according to the invention wherein the foreign nucleic acid molecule is chosen from the group consisting of

a) Nucleic acid molecules, which code at least one antisense RNA, which effects a reduction in the expression of at least one endogenous gene, which codes a Class 3 branching enzyme;

b) Nucleic acid molecules, which by means of a co-suppression effect lead to the reduction in the expression of at least one endogenous gene, which codes a Class 3 branching enzyme;

c) Nucleic acid molecules, which code at least one ribozyme, which splits specific transcripts of at least one endogenous gene, which codes a Class 3 branching enzyme;

d) Nucleic acid molecules, which simultaneously code at least one antisense RNA and at least one sense RNA, wherein the said antisense RNA and the said sense RNA form a double-stranded RNA molecule, which effects a reduction in the expression of at least one endogenous gene, which codes a Class 3 branching enzyme (RNAi technology);

e) Nucleic acid molecules introduced by means of in vivo mutagenesis, which lead to a mutation or an insertion of a heterologous sequence in at least one endogenous gene coding a Class 3 branching enzyme, wherein the mutation or insertion effects a reduction in the expression of a gene coding a Class 3 branching enzyme or results in the synthesis of inactive Class 3 branching enzymes;

f) Nucleic acid molecules, which code an antibody, wherein the antibody results in a reduction in the activity of a Class 3 branching enzyme due to the bonding to a Class 3 branching enzyme.

g) DNA molecules, which contain transposons, wherein the integration of these transposons leads to a mutation or an insertion in at least one endogenous gene coding a Class 3 branching enzyme, which effects a reduction in the expression of at least one gene coding a Class 3 branching enzyme, or results in the synthesis of inactive Class 3 branching enzymes; and/or

h) T-DNA molecules, which, due to insertion in at least one endogenous gene coding a Class 3 branching enzyme, effect a reduction in the expression of at least one gene coding a Class 3 branching enzyme, or result in the synthesis of inactive Class 3 branching enzyme.

The plant cells according to the invention and plants according to the invention can be manufactured by different methods known to the person skilled in the art. These include, for example, the expression of a corresponding antisense RNA or of a double-stranded RNA construct, the provision of molecules or vectors, which impart a cosuppression effect, the expression of a correspondingly constructed ribozyme that splits specific transcripts, which code a Class 3 branching enzyme, or so-called "in vivo mutagenesis". Furthermore, the reduction of the Class 3 branching enzyme activity in plant cells and plants can also be brought about by the simultaneous expression of sense and antisense RNA molecules of the respective target gene to be repressed, preferably of the Class 3 branching enzyme gene.

In addition to this, it is known that *in planta* the formation of double-stranded RNA molecules of promoter sequences can lead *in trans* to methylation and transcriptional inactivation of homologous copies of this promoter (Mette et al., EMBO J. 19, (2000), 5194-5201).

A further possible way in which to reduce the enzymatic activity of proteins in plant cells or plants is the so-called immunomodulation method. It is known that an *in planta* expression of antibodies, which specifically recognise a vegetable protein, results in a reduction of the activity of the proteins concerned in appropriate plant cells due to the formation of a protein antibody complex (Conrad and Manteufel, Trends in Plant Science 6, (2001), 399-402; De Jaeger et al., Plant Molecular Biology 43, (2000), 419-428; Jobling et al., Nature Biotechnology 21, (2003), 77-80).
All these methods are based on the introduction of a foreign or of several foreign nucleic acid molecules into the genome of plant cells or plants and are therefore basically suitable for manufacturing plant cells according to the invention and plants according to the invention.

For inhibiting the expression of genes by means of antisense or cosuppression technology, a DNA molecule can be used, for example, which includes the whole coding sequence for a Class 3 branching enzyme, including any existing flanking sequences, as well as DNA molecules, which include only parts of the coding sequence, whereby these parts must be long enough to produce an antisense effect or a cosuppression effect respectively in the cells. In general, sequences up to a minimum length of 21 bp, preferably a minimum length of at least 100 bp, particularly preferably of at least 500 bp are suitable. For example, the DNA molecules have a length of 21-100 bp, preferably of 100-500 bp, particularly preferably over 500 bp.

The use of DNA sequences, which have a high degree of identity with the endogenous sequences occurring in the plant cells and which code Class 3 branching enzymes, is also suitable for antisense or cosuppression approaches. The minimum identity should be greater than ca. 65%, preferably greater than 80%. The use of sequences with identities of at least 90%, in particular between 95% and 100%, is to be preferred. The meaning of the term "identity" will be defined elsewhere.

Furthermore, the use of introns, i.e. of non-coding areas of genes, which code for Class 3 branching enzymes, is also conceivable for achieving an antisense or a cosuppression effect.

The use of intron sequences for inhibiting the gene expression of genes, which code for starch biosynthesis proteins, has been described in the international patent applications WO97/04112, WO97/04113, WO98/37213, WO98/37214.

The person skilled in the art knows how to achieve an antisense and a cosuppression effect. For example, the method of cosuppression inhibition has been described in Jorgensen (Trends Biotechnol. 8 (1990), 340-344), Niebel et al., (Curr. Top. Microbiol. Immunol. 197 (1995), 91-103), Flavell et al. (Curr. Top. Microbiol. Immunol. 197 (1995), 43-46), Palaqui and Vaucheret (Plant. Mol. Biol. 29 (1995), 149-159), Vaucheret et al., (Mol. Gen. Genet. 248 (1995), 311-317), de Borne et al. (Mol. Gen. Genet. 243 (1994), 613-621).

The expression of ribozymes for reducing the activity of particular enzymes in cells is also known to the person skilled in the art, and is described, for example, in EP-B1 0321201. The expression of ribozymes in vegetable cells has been described, for example, in Feyter et al. (Mol. Gen. Genet. 250, (1996), 329-338).

The reduction of the activity of a Class 3 branching enzyme in plant cells according to the invention and plants according to the invention can also be brought about by the simultaneous expression of sense and antisense RNA molecules (RNAi technology) of the respective target gene to be repressed, preferably of the Class 3 branching enzyme gene.

This can be achieved, for example, by the use of chimeric constructs, which contain "inverted repeats" of the respective target gene or parts of the target gene. In this case, the generic constructs code for sense and antisense RNA molecules of the respective target gene. Sense and antisense RNA are synthesised simultaneously *in planta* as an RNA molecule, wherein sense and antisense RNA are separated from one another by a spacer, and are able to form a double-stranded RNA molecule.

It has been shown that the introduction of inverted repeat DNA constructs into the genome of plant cells or plants is a very effective method of repressing the genes corresponding to the inverted repeat DNA constructs (Waterhouse et al., Proc. Natl. Acad. Sci. USA 95, (1998), 13959-13964; Wang and Waterhouse, Plant Mol. Biol. 43, (2000), 67-82; Singh et al., Biochemical Society Transactions Vol. 28 part 6

(2000), 925- 927; Liu et al., Biochemical Society Transactions Vol. 28 part 6 (2000), 927-929); Smith et al., (Nature 407, (2000), 319-320; international patent application WO99/53050 A1). Sense and antisense sequences of the target gene or the target genes can also be expressed separately from one another by means of similar or different promoters (Nap, J-P et al, 6th International Congress of Plant Molecular Biology, Quebec, 18th-24th June, 2000; Poster S7-27, Presentation Session S7).

The reduction of the activity of a Class 3 branching enzyme in plant cells according to the invention or plants according to the invention can therefore also be achieved by producing double-stranded RNA molecules. In this regard, "inverted repeats" of DNA molecules of Class 3 branching enzyme genes or cDNAs are preferably introduced into the genome of plants, wherein the DNA molecules (Class 3 branching enzyme gene or cDNA or fragments of this gene or cDNA) to be transcribed are under the control of a promoter, which controls the expression of said DNA molecules.

In addition to this, it is known that the formation of double-stranded RNA molecules from promoter DNA molecules in plants *in trans* can lead to methylation and transcriptional inactivation of homologous copies of these promoters, which are to be referred to in the following as target promoters (Mette et al., EMBO J. 19, (2000), 5194-5201).

It is therefore possible to reduce the gene expression of a particular target gene (e.g. branching enzyme Class 3 gene), which is naturally under the control of this target promoter, by deactivating the target promoter.

This means that, in this case, the DNA molecules, which include the target promoters of the genes to be repressed (target genes), in contrast to the original function of promoters in plants, are not used as control elements for the expression of genes or cDNAs, but are themselves used as transcribable DNA molecules.

For the production of double-stranded target promoter RNA molecules *in planta*, which can occur there as RNA hairpin molecules, constructs are preferably used, which contain the "inverted repeats" of the target promoter DNA molecules, wherein the target promoter DNA molecules are under the control of a promoter, which controls the gene expression of said target promoter DNA molecules. These constructs are subsequently introduced into the genome of plants. The expression of

the "inverted repeats" of said target promoter DNA molecules *in planta* leads to the formation of double-stranded target promoter RNA molecules (Mette et al., EMBO J. 19, (2000), 5194-5201). The target promoter can be inactivated by this means.

The reduction of the activity of a Class 3 branching enzyme in plant cells according to the invention and plants according to the invention can therefore also be achieved by the production of double-stranded RNA molecules of promoter sequences of Class 3 branching enzyme genes. In this regard, "inverted repeats" of promoter DNA molecules of Class 3 branching enzyme genes are preferably introduced into the genome of plants, wherein the target promoter DNA molecules (promoter of a Class 3 branching enzyme gene) to be transcribed are under the control of a promoter, which controls the expression of said target promoter DNA molecules.

For inhibiting the expression of genes by means of the simultaneous expression of sense and antisense RNA molecules (RNAi technology), a DNA molecule can be used, for example, which includes the whole coding sequence for a Class 3 branching enzyme, including any existing flanking sequences, as well as DNA molecules, which include only parts of the coding sequence, whereby these parts must be long enough to produce a so-called RNAi effect in the cells. In general, sequences with a minimum length of 40 bp, preferably a minimum length of at least 100 bp, particularly preferably of at least 500 bp are suitable. For example, the DNA molecules have a length of 21-100 bp, preferably of 100-500 bp.

The use of DNA sequences, which have a high degree of identity with the endogenous sequences occurring in the plant cells and which code Class 3 branching enzymes, is also suitable for the simultaneous expression of sense and antisense RNA molecules (RNAi technology). The minimum identity should be greater than ca. 65%, preferably greater than 80%. The use of sequences with identities of at least 90%, in particular between 95% and 100%, is to be particularly preferred.

Furthermore, the reduction of the activity of a Class 3 branching enzyme in plant cells according to the invention and plants according to the invention can also be achieved by so-called "in vivo mutagenesis", in which a hybrid RNA-DNA oligonucleotide

("Chimeroplast") is introduced into plant cells (Kipp, P.B. et al., Poster Session at the "5th International Congress of Plant Molecular Biology, 21st-27th September 1997, Singapore; R. A. Dixon and C.J. Arntzen, meeting report on "Metabolic Engineering in Transgenic Plants", Keystone Symposia, Copper Mountain, CO, USA, TIBTECH 15, (1997), 441-447; international patent application WO 9515972; Kren et al., Hepatology 25, (1997), 1462-1468; Cole-Strauss et al., Science 273, (1996), 1386-1389; Beetham et al., 1999, PNAS 96, 8774-8778).

A part of the DNA components of the RNA-DNA oligonucleotide is homologous to a nucleic acid sequence of an endogenous Class 3 branching enzyme gene, but, in comparison with the nucleic acid sequence of a Class 3 branching enzyme gene, it has a mutation or contains a heterologous region, which is surrounded by the homologous regions.

By base pairing of the homologous regions of the RNA-DNA oligonucleotide and the endogenous nucleic acid molecule followed by homologous recombination, the mutation or heterologous region contained in the DNA components of the RNA-DNA oligonucleotide can be transferred into the genome of a plant cell. This leads to the reduction of the activity of one or more Class 3 branching enzymes.

The person skilled in the art knows that he can achieve the activity of Class 3 branching enzymes by the expression of non-functional derivatives, in particular transdominant mutants, of such proteins, and/or by the expression of antagonists/inhibitors of such proteins.

Antagonist/inhibitors of such proteins include, for example, antibodies, antibody fragments or molecules with similar bonding characteristics. For example, a cytoplasmatic scFv antibody has been used to modulate the activity of the phytochrome A protein in genetically modified tobacco plants (Owen, Bio/Technology 10 (1992), 790-4; Review: Franken, E, Teuschel, U. and Hain, R., Current Opinion in Biotechnology 8, (1997), 411-416; Whitelam, Trends Plant Sci. 1 (1996), 268-272; Conrad and Manteufel, Trends in Plant Science 6, (2001), 399-402; De Jaeger et al., Plant Molecular Biology 43, (2000), 419-428). The reduction of the activity of a branching enzyme in potato plants by expressing a specific antibody has been described by Jobling et al. (Nature Biotechnology 21, (2003), 77-80). Here, the

antibody was provided with a plastidiary target sequence so that the inhibition of proteins localised in plastids was guaranteed.

In conjunction with the present invention, plant cells and plants according to the invention can also be manufactured by the use of so-called insertion mutagenesis (overview article: Thorneycroft et al., 2001, Journal of experimental Botany 52 (361), 1593-1601). Insertion mutagenesis is to be understood to mean particularly the insertion of transposons or so-called transfer DNA (T-DNA) into a gene coding for a Class 3 branching enzyme, whereby, as a result of which, the activity of a Class 3 branching enzyme in the cell concerned is reduced.

The transposons can be both those that occur naturally in the cell (endogenous transposons) and also those that do not occur naturally in said cell but are introduced into the cell (heterologous transposons) by means of genetic engineering methods, such as transformation of the cell, for example. Changing the expression of genes by means of transposons is known to the person skilled in the art. An overview of the use of endogenous and heterologous transposons as tools in plant biotechnology is presented in Ramachandran and Sundaresan (2001, Plant Physiology and Biochemistry 39, 234-252). The possibility of identifying mutants in which specific genes have been inactivated by transposon insertion mutagenesis is presented in an overview by Maes et al. (1999, Trends in Plant Science 4 (3), 90-96). The production of rice mutants with the help of endogenous transposons is described by Hirochika (2001, Current Opinion in Plant Biology 4, 118-122). The identification of maize genes with the help of endogenous retrotransposons is presented, for example, by Hanley et al. (2000, The Plant Journal 22 (4), 557-566). The possibility of manufacturing mutants with the help of retrotransposons and methods of identifying mutants are described by Kumar and Hirochika (2001, Trends in Plant Science 6 (3), 127-134). The activity of technological transposons in different species has been described both for dicotyledonous and for monocotyledonous plants: e.g. for rice (Greco et al., 2001, Plant Physiology 125, 1175-1177; Liu et al., 1999, Molecular and General Genetics 262, 413-420; Hiroyuki et al., 1999, The Plant Journal 19 (5), 605-613; Jeon-und-Gynheung, 2001, Plant Science 161, 211-219), barley (2000, Koprek et al., The Plant Journal 24 (2), 253-263) *Arabidopsis thaliana* (Aarts et al., 1993,

Nature 363, 715-717, Schmidt und Willmitzer, 1989, Molecular and General Genetics 220, 17-24; Altmann et al., 1992, Theoretical and Applied Gentics 84, 371-383; Tissier et al., 1999, The Plant Cell 11, 1841-1852), tomato (Belzile und Yoder, 1992, The Plant Journal 2 (2), 173-179) and potato (Frey et al., 1989, Molecular and General Genetics 217, 172-177; Knapp et al., 1988, Molecular and General Genetics 213, 285-290).

Basically, the plant cells according to the invention and the plants according to the invention can be manufactured both with the help of homologous and heterologous transposons, whereby the use of homologous transposons is also to be understood to mean those, which are naturally present in the corresponding wild type plant genome.

T-DNA insertion mutagenesis is based on the fact that certain sections (T-DNA) of Ti plasmids from *Agrobacterium* can integrate into the genome of vegetable cells. The place of integration in the vegetable chromosome is not defined, but can take place at any point. If the T-DNA integrates into a part of the chromosome, which constitutes a gene function, then this can lead to a change in the gene expression and thus also to a change in the activity of a protein coded by the gene concerned. In particular, the integration of a T-DNA into the coded area of a protein often leads to the corresponding protein no longer being able to be synthesised at all, or no longer synthesised in active form, by the cell concerned. The use of T-DNA insertions for producing mutants is described, for example, for *Arabidopsis thaliana* (Krysan et al., 1999, The Plant Cell 11, 2283-2290; Atipiroz-Leehan and Feldmann, 1997, Trends in genetics 13 (4), 152-156; Parinov and Sundaresan, 2000, Current Opinion in Biotechnology 11, 157-161) and rice (Jeon and An, 2001, Plant Science 161, 211-219; Jeon et al., 2000, The Plant Journal 22 (6), 561-570). Methods for identifying mutants, which have been produced with the help of T-DNA insertion mutagenesis, are described, amongst others, by Young et al., (2001, Plant Physiology 125, 513-518), Parinov et al. (1999, The Plant cell 11, 2263-2270), Thorneycroft et al. (2001, Journal of Experimental Botany 52, 1593-1601), and McKinney et al. (1995, The Plant Journal 8 (4), 613-622).

T-DNA mutagenesis is basically suitable for the production of the plant cells and plants according to the invention, which have a reduced activity of a Class 3 branching enzyme.

Surprisingly, it has been found that plant cells according to the invention and plants according to the invention synthesise a modified starch in comparison with starch of corresponding wild type plant cells or wild type plants that have not been genetically modified.

The plant cells according to the invention and plants according to the invention synthesise a modified starch, which in its physical-chemical characteristics, in particular the amylose content or the amylose/amylopectin ratio, the degree of branching, the average chain length, the side chain distribution, the viscosity behaviour, the gelling strength, the starch grain size and/or the starch grain morphology, is changed in comparison with the synthesised starch in wild type plant cells or plants, so that this is better suited for special applications.

It was surprisingly found that plant cells or plants of the invention synthesize a modified starch having decreased phosphate content. So far known plants with a reduced activity of a branching enzyme (Class 1 and/or Class 2) did show an increased phosphate content.

The present invention therefore also includes plant cells according to the invention and plants according to the invention, which synthesise a modified starch.

In a preferred embodiment of the invention, the plant cells according to the invention or the plant according to the invention synthesize a starch with a decreased phosphate content in comparison with corresponding starch isolated from wild type plant cells or wild type plants that have not been genetically modified. Preferably the plant cells according to the invention or the plants according to the invention synthesize a starch having a total phosphate content that is decreased by at least 10%, more preferably by at least 15% and particular preferably by at least 20% in comparison with starch isolated from corresponding wild type plant cells or wild type

plants that have not been genetically modified. Especially preferably the total phosphate content of starch isolated from plant cells of the invention or plants of the invention is decreased by 14% to 22% in comparison with starch isolated from corresponding wild type plant cells or wild type plants that have not been genetically modified.

In respect with C-6-phoaphate content the plant cells according to the invention or the plants according to the invention synthesize a starch having a C-6-phosphate content that is decreased by at least 15%, more preferably by at least 19% and particular preferably by at least 25% in comparison with starch isolated from corresponding wild type plant cells or wild type plants that have not been genetically modified. Especially preferably the C-6-phoaphate content of starch isolated from plant cells of the invention or plants of the invention is decreased by 15%% to 27% in comparison with starch isolated from corresponding wild type plant cells or wild type plants that have not been genetically modified.

Methods for the determination of total phosphate or C-6-phosphate content in starches are well known by a person skilled in the art. Preferred methods for the determination of total or C-6-phosphate content in starches to be used in combination with the present invention are described below in the section "general methods" (Starch analysis, e) Analysis of the side-chain distribution of the amylopectin by means of ion-exchange chromatography).

In a further prefered embodiment embodiment of the invention, the plant cells according to the invention or the plants according to the invention synthesize a starch which has has an altered viscosity behaviour in comparison with starch isolated from corresponding wild type plant cells or wild type plants that have not been genetically modified. Plant cells of the invention or plants of the invention synthesize a starch which has a decreased maximum viscosity, a decreased minimum viscosity and/or a decreased final viscosity in comparison with starch isolated from corresponding wild type plant cells or wild type plants that have not been genetically modified.
The maximum viscosity of starch isolated from plant cells of the invention or plants of the invention is preferably decreased by at least 8% and more preferably by at least

16% in comparison with starch isolated from corresponding wild type plant cells or wild type plants that have not been genetically modified. Especially preferably the maximum viscosity of starch isolated from plant cells of the invention or plants of the invention is decreased by 8% to 16%_in comparison with starch isolated from corresponding wild type plant cells or wild type plants that have not been genetically modified.

The minimum viscosity of starch isolated from plant cells of the invention or plants of the invention is preferably decreased by at least 10%, more preferably by at least 15% and particularly preferably by at lest 25% in comparison with starch isolated from corresponding wild type plant cells or wild type plants that have not been genetically modified. Especially preferably the minimum viscosity of starch isolated from plant cells of the invention or plants of the invention is decreased by 15% to 25% in comparison with starch isolated from corresponding wild type plant cells or wild type plants that have not been genetically modified.

The final viscosity of starch isolated from plant cells of the invention or plants of the invention is preferably decreased by at least 5% and more preferably by at least 10% in comparison with starch isolated from corresponding wild type plant cells or wild type plants that have not been genetically modified. Particularly preferably the minimum viscosity of starch isolated from plant cells of the invention or plants of the invention is decreased by 5% to 10% in comparison with starch isolated from corresponding wild type plant cells or wild type plants that have not been genetically modified.

It has further been found, that starch isolated from plant cells of the invention or plants of the invention shows an increased gelling strength in comparison with starch isolated from corresponding wild type plant cells or wild type plants that have not been genetically_modified.

The present invention therefore also comprises plant cells of the invention or plants of the invention that synthesize a starch with an increased gel strength in comparison with starch isolated from corresponding wild type plant cells or wild type plants that have not been genetically modified. Preferably plant cells of the invention or plants of the invention synthesize a starch which shows a gel strength which is increased by at

least 20%, more preferably by at least 30% and particular preferably by at least 35% in comparison with starch isolated from corresponding wild type plant cells or wild type plants that have not been genetically modified. Especially preferably the gel strength of starch isolated from plant cells of the invention or plants of the invention is increased by 27% to 38% in comparison with starch isolated from corresponding wild type plant cells or wild type plants that have not been genetically modified.

Methods for the determination of viscosity behaviour or gelling properties of starches are well known by a person skilled in the art. Preferred methods for the determination of viscosity behaviour or gelling properties of starches to be used in combination with the present invention are described below in the section "general methods".

Furthermore it was surprisingly found that starch, isolated from plant cells of the invention or plants of the invention shows an altered side chain distribution pattern in the amylopectin fraction in comparison with the amylopectin fraction from starch isolated from corresponding wild type plant cells or wild type plants that have not been genetically modified.

In a further embodiment of the invention, plant cells according to the invention or the plants according to the invention synthesize a starch with an altered short-side-chain distribution pattern in the amylopectin fraction in comparison with the amylopectin fraction from starch isolated from corresponding wild type plant cells or wild type plants that have not been genetically modified. Preferably plant cells according to the invention or the plants according to the invention synthesize a starch wherein the short-side-chains in the amylopectin fraction having a degree of polymerization (DP) of 6 and/or a DP of 7 is increased in comparison with the amylopectin fraction from starch isolated from corresponding wild type plant cells or wild type plants that have not been genetically modified. More preferably the amylopectin fraction of starch isolated form plant cells according to the invention or plants according to the invention synthesize a starch wherein short-side-chains with a DP 6 is increased by at least 15%, particularly preferably by at least 20%, especially particularly by at least 25% and/or the short-side-chains with a DP 7 are increased by at least 2%, particularly preferably by at least 4%, especially preferably by at least 8% in

comparison with the amylopectin fraction from starch isolated from corresponding wild type plant cells or wild type plants that have not been genetically modified.

In a further preferred embodiment of the invention the plant cells according to the invention or the plants according to the invention synthesize a starch wherein the short-side-chains of DP 6 in the amylopectin fraction is increased by 17% to 29% and/or the side chains of DP 7 in the amylopectin fraction is increased by 2% to 9% in comparison with the amylopectin fraction from starch isolated from corresponding wild type plant cells or wild type plants that have not been genetically modified.

In conjunction with the present invention, the term "short-side-chain" shall mean alpha-1,6-linked side-chains in the starch molecule having a degree of polymerization between DP 6 and DP 34.

Methods for the quantification of short-side-chains having a specified DP in the amylopectin fraction are well known by the person skilled in the art. Preferred methods for the quantification of side-chains having a specified DP, suitable to be used in combination with the present invention are described below in the section "general methods (Analysis of the side-chain distribution of the amylopectin by means of ion-exchange chromatography).

Furthermore it was found that the amylopectin fraction of starch, isolated from the plant cells according to the invention or the plants according to the invention shows an altered total-side-chain distribution.

Therefore, further embodiments of the present invention are the plant cells according to the invention or the plants according to the invention which synthesize a starch wherein the groups of total-side-chains in the amylopectin fraction characterized by the following ranges:

a) DP up to 11,

b) DP 12 to DP 19,

c) DP 20 to Dp 25 and/or

d) DP 26 to DP 31

is/are increased and/or the groups of total-side-chains in the amylopectin fraction characterized by the following ranges:

a) DP 38 to DP 43
b) DP 44 to DP 49
c) DP 50 to DP 56
d) DP 57 to DP 62 and/or
e) DP 63 to DP 123

Is/are decreased in comparison with the amylopectin fraction from starch isolated from corresponding wild type plant cells or wild type plants that have not been genetically modified.

In conjunction with the present invention, the term "total-side-chains" shall mean alpha-1,6-linked side-chains in the starch molecule having a degree of polymerization up to DP 123. A group of total-side-chains consists of all side-chains spanning a defined DP range (e.g. DP up to 11, DP 12 to DP 19, DP 20 to Dp 25, DP 26 to DP 31, DP 38 to DP 43, DP 44 to DP 49, DP 50 to DP 56, DP 57 to DP 62, DP 63 to DP 123).

Methods for the quantification of groups of total-side-chains spanning ranges of side-chains with a specified DP in the amylopectin fraction are well known by the person skilled in the art. Preferred methods for the quantification groups of total-side-chains, suitable to be used in combination with the present invention are described below in example 5d).

Further embodiments of the invention are the plant cells according to the invention or the plants according to the invention which synthesize a starch having a decreased peak onset Temperature (T_0), a decreased peak temperature (T Peak) and an increased delta H (dH) when analyzed by differential scanning calorimetrie (DSC) in comparison to starch isolated from corresponding wild type plant cells or wild type plants that have not been genetically modified.

Methods for the analysis of starch by DSC are well known by a person skilled in the art. Prefered Methods for DSC analysis suitable to be used in combination with the

present invention are described below in the section "general methods" (DSC-analysis („Differential Scanning Calorimetry").

Furthermore, genetically modified plants, which contain the plant cells according to the invention, are also the subject matter of the invention. Plants of this type can be produced from plant cells according to the invention by regeneration.
In principle, the plants according to the invention can be plants of any plant species, i.e. both monocotyledonous and dicotyledonous plants. Preferably they are useful plants, i.e. plants, which are cultivated by people for the purposes of food or for technical, in particular industrial purposes.

In a further preferred embodiment, the plant according to the invention is a starch-storing plant.

In a further preferred embodiment, the present invention relates to starch-storing plants according to the invention of the genus *Solanum*, in particular *Solanum tuberosum*.

The term "starch-storing plants" includes all plants with starch-storing plant parts such as, for example, maize, rice, wheat, rye, oat, barley, cassava, potato, sago, mung bean, pea or sorghum. Preferred starch-storing plant parts are, for example, tubers, storage roots and grains containing an endosperm; tubers are particularly preferred.

In conjunction with the present invention, the term "potato plant" or "potato" means plant species of the genus *Solanum*, in particular tuber-producing species of the genus *Solanum* and especially *Solanum tuberosum*.

The present invention also relates to propagation material of plants according to the invention containing a plant cell according to the invention.

Here, the term "propagation material" includes those constituents of the plant that are suitable for producing offspring by vegetative or sexual means. Cuttings, callus

cultures, rhizomes or tubers, for example, are suitable for vegetative propagation. Other propagation material includes, for example, fruits, seeds, seedlings, protoplasts, cell cultures, etc. Preferably, the propagation material is seeds and particularly preferably tubers.

In a further embodiment, the present invention relates to harvestable plant parts of plants according to the invention such as fruits, storage roots, roots, blooms, buds, shoots or stems, preferably seeds or tubers, wherein these harvestable parts contain at least one plant cell according to the invention.

Furthermore, the present invention also relates to a method for the manufacture of a plant according to the invention, wherein

a) a plant cell is genetically modified, whereby the genetic modification leads to the reduction of the activity of a Class 3 vegetable branching enzyme in comparison with corresponding wild type plant cells that have not been genetically modified;
b) a plant is regenerated from plant cells from Step a); and
c) if necessary, further plants are produced with the help of the plants according to Step b).

The genetic modification introduced into the plant cell according to Step a) can basically be any type of genetic modification, which leads to the reduction of the activity of a Class 3 branching enzyme.

The regeneration of the plants according to Step (b) can be carried out using methods known to the person skilled in the art (e.g. described in "Plant Cell Culture Protocols", 1999, edt. by R.D. Hall, Humana Press, ISBN 0-89603-549-2).

The production of further plants according to Step (c) of the method according to the invention can be carried out, for example, by vegetative propagation (for example using cuttings, tubers or by means of callus culture and regeneration of whole plants) or by sexual propagation. Here, sexual propagation preferably takes place under

controlled conditions, i.e. selected plants with particular characteristics are crossed and propagated with one another.

In a preferred embodiment of the method according to the invention, the genetic modification consists in the introduction of a foreign nucleic acid molecule into the genome of the plant cell, wherein the presence or the expression of said foreign nucleic acid molecule leads to a reduced activity of a Class 3 branching enzyme in the cell.

The statements made in conjunction with plant cells according to the invention and plants according to the invention apply with regard to the "introduction of a foreign nucleic acid molecule".

In a further preferred embodiment, the method according to the invention is used for producing potato plants according to the invention.

In a further preferred embodiment of the method according to the invention, the foreign nucleic acid molecule is chosen from the group consisting of

a) Nucleic acid molecules, which code a protein with the amino acid sequence given under Seq ID NO 4;
b) Nucleic acid molecules, which code a protein, the amino acid sequence of which has an identity of at least 50% with the amino acid sequence given under SEQ ID NO: 4;
c) Nucleic acid molecules, which include the nucleotide sequence shown under Seq ID NO. 3 or a complimentary sequence;
d) Nucleic acid molecules, the nucleic acid sequence of which has an identity of at least 50% with the nucleic acid sequences described under a) or c);
e) Nucleic acid molecules, which hybridise with at least one strand of the nucleic acid molecules described under a) or c) under stringent conditions;
f) Nucleic acid molecules, the nucleotide sequence of which deviates from the sequence of the nucleic acid molecules identified under a), b), c), d), e) or f) due to the degeneration of the genetic code; and

g) Nucleic acid molecules, which represent fragments, allelic variants and/or derivatives of the nucleic acid molecules identified under a), b), c), d), e) or f).

In a further preferred embodiment of the method according to the invention, the foreign nucleic acid molecule is chosen from the group consisting of

a) Nucleic acid molecules, which code at least one antisense RNA, which effects a reduction in the expression of at least one endogenous gene, which codes a Class 3 branching enzyme;

b) Nucleic acid molecules, which by means of a co-suppression effect lead to the reduction in the expression of at least one endogenous gene, which codes a Class 3 branching enzyme;

c) Nucleic acid molecules, which code at least one ribozyme, which splits specific transcripts of at least one endogenous gene, which codes a Class 3 branching enzyme;

d) Nucleic acid molecules, which simultaneously code at least one antisense RNA and at least one sense RNA, wherein the said antisense RNA and the said sense RNA form a double-stranded RNA molecule, which effects a reduction in the expression of at least one endogenous gene, which codes a Class 3 branching enzyme (RNAi technology);

e) Nucleic acid_molecules introduced by means of in vivo mutagenesis, which lead to a mutation or an insertion of a heterologous sequence in at least one endogenous gene coding a Class 3 branching enzyme, wherein the mutation or insertion effects a reduction in the expression of a gene coding a Class 3 branching enzyme or results in the synthesis of inactive Class 3 branching enzymes;

f) Nucleic acid molecules, which code an antibody, wherein the antibody results in a reduction in the activity of a Class 3 branching enzyme due to the bonding to a Class 3 branching enzyme.

g) Nucleic acid molecules, which contain transposons, wherein the integration of these transposons leads to a mutation or an insertion in at least one endogenous gene coding a Class 3 branching enzyme, which effects a reduction in the expression of at least one gene coding a Class 3 branching

enzyme, or results in the synthesis of inactive Class 3 branching enzymes; and/or

h) T-DNA molecules, which, due to insertion in at least one endogenous gene coding a Class 3 branching enzyme, effect a reduction in the expression of at least one gene coding a Class 3 branching enzyme, or result in the synthesis of inactive Class 3 branching enzyme.

In a further embodiment of the method according to the invention, the genetically modified plants according to the invention synthesise a modified starch in comparison with corresponding wild type plants that have not been genetically modified.

In a further embodiment of the method according to the invention, the method according to the invention is used to manufacture plants according to the invention.

The present invention also relates to the plants obtainable by the method according to the invention.

It is also an object of the present invention to provide means such as DNA molecules, for example, for the production of plant cells according to the invention and plants according to the invention, which synthesise a modified starch in comparison with modified wild type plant cells or wild type plants that have not been genetically modified.

The present invention therefore also relates to nucleic acid molecules coding for a protein with the enzymatic activity of a Class 3 branching enzyme, chosen from the group consisting of

a) Nucleic acid molecules, which code a protein with the amino acid sequence given under Seq ID NO 4;

b) Nucleic acid molecules, which code a protein, which includes the amino acid sequence, which is coded by the insertion in plasmid DSM 15926;

c) Nucleic acid molecules, which code a protein, the sequence of which has an identity of at least 70% with the amino acid sequence given under SEQ ID NO 4;

d) Nucleic acid molecules, which code a protein, the sequence of which has an identity of at least 70% with the amino acid sequence, which is coded by the insertion in plasmid DSM 15926;

e) Nucleic acid molecules, which include the nucleotide sequence shown under Seq ID NO 3 or a complimentary sequence;

f) Nucleic acid molecules, which include the nucleotide sequence of the insertion contained in plasmid DSM 15926;

g) Nucleic acid molecules, which have an identity of at least 70% with the nucleic acid sequences described under a), b), d) or e);

h) Nucleic acid molecules, which hybridise with at least one strand of the nucleic acid molecules described under a), b), d), e) or f) under stringent conditions;

i) Nucleic acid molecules, the nucleotide sequence of which deviates from the sequence of the nucleic acid molecules identified under a), b), e) or f) due to the degeneration of the genetic code; and

j) Nucleic acid molecules, which represent fragments, allelic variants and/or derivatives of the nucleic acid molecules identified under a), b), c), d), e), f), g), h) or i).

The amino acid sequence shown in SEQ ID NO 4 codes a protein with the activity of a Class 3 branching enzyme from *Solanum tuberosum*.

The proteins coded from the different varieties of nucleic acid molecules according to the invention have certain common characteristics. These can include, for example, biological activity, molecular weight, immunological reactivity, conformation etc, as well as physical characteristics such as, for example, the running behaviour in gel electrophoresis, chromatographic behaviour, sedimentation coefficients, solubility, spectroscopic characteristics, stability; optimum pH, optimum temperature etc.
The molecular weight of the Class 3 branching enzyme from *Solanum tuberosum* derived from the amino acid sequence shown under SEQ ID NO 4 is ca. 103 kDa. The derived molecular weight of a protein according to the invention therefore

preferably lies in the range from 85 kDa to 120 kDa, preferably in the range from 95 kDa to 110 kDa and particularly preferably from ca. kDa 100 to 105 kDa.

The present invention relates to nucleic acid molecules, which code a protein with the enzymatic activity of a Class 3 branching enzyme, wherein the coded protein has an identity of at least 70%, preferably of at least 80%, particularly preferably of at least 90% and especially preferably of 95% with the amino acid sequence specified under SEQ ID NO 4.

A plasmid containing a cDNA, which codes a Class 3 branching enzyme from *Solanum tuberosum*, was deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, 38124 Braunschweig, Germany, in accordance with the Budapest Treaty on 15th September 2003 under the number DSM 15926. The amino acid sequence shown SEQ ID NO 4 can be derived from the coding region of the cDNA sequence integrated in plasmid DSM 15926 and codes for a Class 3 branching enzyme from *Solanum tuberosum*. The present invention therefore also relates to nucleic acid molecules, which code a protein with the enzymatic activity of a Class 3 branching enzyme, which includes the amino acid sequence, which is coded by the insertion in plasmid DSM 15926, wherein the coded protein has an identity of at least 70%, preferably of at least 80%, particularly preferably of at least 90% and especially preferably of 95% with the amino acid sequence, which can be derived from the insertion in DSM 15926.

The nucleic acid sequence shown SEQ ID NO 3 is a cDNA sequence, which includes the coding region for a Class 3 branching enzyme from *Solanum tuberosum*.
The present invention therefore also relates to nucleic acid molecules, which code a Class 3 branching enzyme and the coding region of the nucleotide sequence shown under Seq ID NO 3 or a complimentary sequence, nucleic acid molecules, which include the coding region of the nucleotide sequence of the insertion contained in plasmid DSM 15926 and nucleic acid molecules, which have an identity of at least 70%, preferably of at least 80%, particularly preferably of at least 90% and especially preferably of at least 95% with the said nucleic acid molecules.

With the help of the sequence information of the nucleic acid molecule according to the invention or with the help of the nucleic acid molecule according to the invention, it is now possible for the person skilled in the art to isolate homologous sequences from other plant species, preferably from starch-storing plants, preferably from plant species of the genus *Solanum*, particularly preferably from *Solanum tuberosum*. This can be carried out, for example, with the help of conventional methods such as the examination of cDNA or genomic banks with suitable hybridisation samples. The person skilled in the art knows that homologous sequences can also be isolated with the help of (degenerated) oligonucleotides and the use of PCR-based methods.

The examination of databases, such as are made available, for example, by EMBL (http://www.ebi.ac.uk/Tools/index.htm) or NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/), can also be used for identifying homologous sequences, which code for a Class 3 branching enzyme. In this case, one or more sequences are specified as a so-called query. This query sequence is then compared by means of statistical computer programs with sequences, which are contained in the selected databases. Such database queries (e.g. blast or fasta searches) are known to the person skilled in the art and can be carried out by various providers.

If such a database query is carried out, e.g. at the NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/), then the standard settings, which are specified for the particular comparison inquiry, should be used. For protein sequence comparisons (blastp), these are the following settings: Limit entrez = not activated; Filter = low complexity activated; Expect value = 10; word size = 3; Matrix = BLOSUM62; Gap costs: Existence = 11, Extension = 1.

For nucleic acid sequence comparisons (blastn), the following parameters must be set: Limit entrez = not activated; Filter = low complexity activated; Expect value = 10; word size = 11.

With such a database search, the sequences described in the present invention can be used as a query sequence in order to identify further nucleic acid molecules and/or proteins, which code a Class 3 branching enzyme.

With the help of the described methods, it is also possible to identify and/or isolate nucleic acid molecules according to the invention, which hybridise with the sequence specified under SEQ ID NO 3 and which code a Class 3 branching enzyme.

Within the framework of the present invention, the term "hybridising" means hybridisation under conventional hybridisation conditions, preferably under stringent conditions such as, for example, are described in Sambrock et al., Molecular Cloning, A Laboratory Manual, 2nd Ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). Particularly preferably, "hybridising" means hybridisation under the following conditions:

Hybridisation buffer:

2xSSC; 10xDenhardt solution (Ficoll 400+PEG+BSA; Ratio 1:1:1); 0.1% SDS; 5 mM EDTA; 50 mM Na_2HPO_4; 250 µg/ml herring sperm DNA; 50 µg/ml tRNA; or

25 M sodium phosphate buffer pH 7.2; 1 mM EDTA; 7% SDS

Hybridisation temperature: T=65 to 68°C

Wash buffer: 0.2xSSC; 0.1% SDS

Wash temperature: T=65 to 68°C.

In principle, nucleic acid molecules, which hybridise with the nucleic acid molecules according to the invention, can originate from any plant species, which expresses an appropriate protein, preferably they originate from starch-storing plants, preferably from species of the genus *Solanum*, particularly preferably from *Solanum tuberosum*. Nucleic acid molecules, which hybridise with the molecules according to the invention, can, for example, be isolated from genomic or from cDNA libraries. The identification and isolation of nuclear acid molecules of this type can be carried out using the nucleic acid molecules according to the invention or parts of these molecules or the reverse complements of these molecules, e.g. by means of hybridisation according to standard methods (see, for example, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) or by amplification using PCR.

Nucleic acid molecules, which exactly or essentially have the nucleotide sequence specified under SEQ ID NO 3 or parts of this sequence, can be used as hybridisation samples. The fragments used as hybridisation samples can also be synthetic fragments or oligonucleotides, which have been manufactured using established synthesising techniques and the sequence of which corresponds essentially with that of a nucleic acid molecule according to the invention. If genes have been identified and isolated, which hybridise with the nucleic acid sequences according to the invention, then a determination of this sequence and an analysis of the

characteristics of the proteins coded by this sequence should be carried out in order to establish whether a Class 3 branching enzyme is involved. Homology comparisons on the level of the nucleic acid or amino acid sequence and a determination of the enzymatic activity are particularly suitable for this purpose. As described above, the activity of a Class 3 branching enzyme can take place by expression in E. coli strains, which themselves do not express an active branching enzyme (Kiel et al., 1987 Mol. Gen. Genet 207: 294-301); Guan et al., 1995, Proc. Natl. Acad. Sci. 92, 964-967).

The molecules hybridising with the nucleic acid molecules according to the invention particularly include fragments, derivatives and allelic variants of the nucleic acid molecules according to the invention, which code a Class 3 branching enzyme from plants, preferably from starch-storing plants, preferably from plant species of the genus *Solanum*, particularly preferably from *Solanum tuberosum*. In conjunction with the present invention, the term "derivative" means that the sequences of these molecules differ at one or more positions from the sequences of the nucleic acid molecules described above and have a high degree of identity with these sequences. Here, the deviation from the nucleic acid molecules described above can have come about, for example, due to deletion, addition, substitution, insertion or recombination.

Furthermore, identity means that functional and/or structural equivalence exists between the nucleic acid molecules concerned or the proteins coded by them. The nucleic acid molecules, which are homologous to the molecules described above and constitute derivatives of these molecules, are generally variations of these molecules, which constitute modifications, which execute the same biological function. At the same time, the variations can occur naturally, for example they can be sequences from other plant species, or they can be mutations, wherein these mutations may have occurred in a natural manner or have been introduced by objective mutagenesis. The variations can also be synthetically manufactured sequences. The allelic variants can be both naturally occurring variants and also synthetically manufactured variants or variants produced by recombinant DNA techniques. Nucleic acid molecules, which deviate from nucleic acid molecules according to the invention due to degeneration of the genetic code, constitute a special form of derivatives.

The proteins coded from the different derivatives of nucleic acid molecules according to the invention have certain common characteristics. These can include, for example, biological activity, substrate specificity, molecular weight, immunological reactivity, conformation etc, as well as physical characteristics such as, for example, the running behaviour in gel electrophoresis, chromatographic behaviour, sedimentation coefficients, solubility, spectroscopic characteristics, stability; optimum pH, optimum temperature etc.

The nucleic acid molecules according to the invention can be any nucleic acid molecules, in particular DNA or RNA molecules, for example cDNA, genomic DNA, mRNA etc. They can be naturally occurring molecules or molecules manufactured by genetic or chemical synthesis methods. They can be single-stranded molecules, which either contain the coding or the non-coding strand, or double-stranded molecules.

Furthermore, the present invention relates to nucleic acid molecules of at least 21, preferably more than 50 and particularly preferably more than 200 nucleotides length, which specifically hybridise with at least one nucleic acid molecule according to the invention. Here, specifically hybridise means that these molecules hybridise with nucleic acid molecules, which code a protein according to the invention, but not with nucleic acid molecules, which code other proteins. In particular, the invention relates to such nucleic acid molecules, which hybridise with transcripts of nucleic acid molecules according to the invention and, as a result, can hinder their translation. Such nucleic acid molecules, which specifically hybridise with the nucleic acid molecules according to the invention, can, for example, be constituents of antisense, RNAi or cosuppression constructs or ribozymes, or can be used as primers for PCR amplification.

In conjunction with the present invention, the term "identity" means a sequence identity over the whole length of the coding region of at least 60%, in particular an identity of at least 70%, preferably greater than 80%, particularly preferably greater than 90% and especially of at least 95%. In conjunction with the present invention, the term "identity" is to be understood to mean the number of amino

acids/nucleotides (identity) corresponding with other proteins/nucleic acids, expressed as a percentage. Identity is preferably determined by comparing the Seq. ID NO 4 or SEQ ID NO 3 with other proteins/nucleic acids with the help of computer programs. If sequences that are compared with one another have different lengths, the identity is to be determined in such a way that the number of amino acids, which have the shorter sequence in common with the longer sequence, determines the percentage quotient of the identity. Preferably, identity is determined by means of the computer program ClustalW, which is well known and available to the public (Thompson et al., Nucleic Acids Research 22 (1994), 4673-4680). ClustalW is made publicly available by Julie Thompson (Thompson@EMBL-Heidelberg.DE) and Toby Gibson (Gibson@EMBL-Heidelberg.DE), European Molecular Biology Laboratory, Meyerhofstrasse 1, D 69117 Heidelberg, Germany. ClustalW can also be downloaded from different Internet sites, including the IGBMC (Institut de Génétique et de Biologie Moléculaire et Cellulaire, B.P.163, 67404 Illkirch Cedex, France; ftp://ftp-igbmc.u-strasbg.fr/pub/) and the EBI (ftp://ftp.ebi.ac.uk/pub/software/) as well as from all mirrored Internet sites of the EBI (European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SD, UK).

Preferably, Version 1.8 of the ClustalW computer program is used to determine the identity between proteins according to the invention and other proteins. In doing so, the following parameters must be set: KTUPLE=1, TOPDIAG=5, WINDOW=5, PAIRGAP=3, GAPOPEN=10, GAPEXTEND=0.05, GAPDIST=8, MAXDIV=40, MATRIX=GONNET, ENDGAPS(OFF), NOPGAP, NOHGAP.

Preferably, Version 1.8 of the ClustalW computer program is used to determine the identity between the nucleotide sequence of the nucleic acid molecules according to the invention, for example, and the nucleotide sequence of other nucleic acid molecules. In doing so, the following parameters must be set:
KTUPLE=2, TOPDIAGS=4, PAIRGAP=5, DNAMATRIX:IUB, GAPOPEN=10, GAPEXT=5, MAXDIV=40, TRANSITIONS: unweighted.

Basically, nucleic acid molecules according to the invention, can originate from any plant, preferably they originate from starch-storing plants, preferably from plant species of the genus *Solanum*, particularly preferably from *Solanum tuberosum*.

Furthermore, the invention relates to vectors, in particular plasmids, cosmids, viruses, bacteriophages and other common vectors in genetic engineering, which contain the nucleic acid molecules according to the invention described above.

In a preferred embodiment, the nucleic acid molecules according to the invention contained in the vectors are linked with regulatory sequences, which guarantee expression in prokaryontic or eukaryontic cells. Here, the term "expression" can mean both transcription as well as transcription and translation. In this case, the nucleic acid molecules according to the invention can be present in "sense" orientation and/or in "antisense" orientation to the regulatory sequences.

Regulatory sequences for expression in prokaryontic organisms, e.g. *E. coli*, and in eukaryontic organisms are adequately described in the literature, in particular those for expression in yeast such as *Saccharomyces cerevisiae*, for example. An overview of different expression systems for proteins in different host organisms can be found, for example, in Methods in Enzymology 153 (1987), 383-516 and in Bitter et al. (Methods in Enzymology 153 (1987), 516-544).

For expressing the nucleic acid molecules, which code a Class 3 branching enzyme, in sense and/or antisense orientation in vegetable cells, these are preferably linked with regulatory DNA sequences, which guarantee transcription in vegetable cells. In particular, these include promoters. In general, any promoter that is active in vegetable cells is eligible for expression.

At the same time, the promoter can be chosen so that expression takes place constitutively or only in a certain tissue, at a certain stage of the plant development or at a time determined by external influences. The promoter can be homologous or heterologous both with respect to the plant and with respect to the nucleic acid molecule.

Suitable promoters are, for example, the promoter of the 35S RNA of the cauliflower mosaic virus and the ubiquitin promoter from maize for constitutive expression, the patatin promoter B33 (Rocha-Sosa et al., EMBO J. 8 (1989), 23-29) for tuber-specific expression in potatoes or a promoter, which only ensures expression in photosynthetically active tissues, e.g. the ST-LS1 promoter (Stockhaus et al., Proc.

Natl. Acad. Sci. USA 84 (1987), 7943-7947; Stockhaus et al., EMBO J. 8 (1989), 2445-2451) or, for endosperm-specific expression of the HMG promoter from wheat, the USP promoter, the phaseolin promoter, promoters of zein genes from maize (Pedersen et al., Cell 29 (1982), 1015-1026; Quatroccio et al., Plant Mol. Biol. 15 (1990), 81-93), glutelin promoter (Leisy et al., Plant Mol. Biol. 14 (1990), 41-50; Zheng et al., Plant J. 4 (1993), 357-366; Yoshihara et al., FEBS Lett. 383 (1996), 213-218) or shrunken-1 promoter (Werr et al., EMBO J. 4 (1985), 1373-1380). However, promoters can also be used, which are only activated at a time determined by external influences (see for example WO 9307279). Promoters of heat-shock proteins, which allow simple induction, can be of particular interest here. Furthermore, seed-specific promoters can be used, such as the USP promoter from *Vicia faba*, which guarantees seed-specific expression in *Vicia faba* and other plants (Fiedler et al., Plant Mol. Biol. 22 (1993), 669-679; Bäumlein et al., Mol. Gen. Genet. 225 (1991), 459-467).

Furthermore, a termination sequence (polyadenylation signal) can be present, which is used for adding a poly-A tail to the transcript. A function in the stabilisation of the transcripts is ascribed to the poly-A tail. Elements of this type are described in the literature (cf. Gielen et al., EMBO J. 8 (1989), 23-29) and can be exchanged at will.

In a further embodiment, the present invention relates to vectors, which contain DNA molecules, which code at least one antisense RNA, which effects a reduction in the expression of at least one endogenous gene, which codes a Class 3 branching enzyme.

In a further special embodiment, the present invention relates to vectors, which contain DNA molecules, which by means of a cosuppression effect lead to a reduction in the expression of at least one endogenous gene, which codes a Class 3 branching enzyme.

In a further embodiment, the present invention relates to vectors, which contain DNA molecules, which code at ~~least one ribozyme, which~~ splits specific transcripts of at least one endogenous gene, which codes a Class 3 branching enzyme.

In a further embodiment, the present invention relates to vectors, which contain DNA molecules, which simultaneously code at least one antisense RNA and at least one sense RNA, wherein the said antisense RNA and the said sense RNA form a double-stranded RNA molecule, which effects a reduction in the expression of at least one endogenous gene, which codes a Class 3 branching enzyme (RNAi technology).

A further subject of the present invention is a host cell, in particular a prokaryontic or eukaryontic cell, which is genetically modified with a nucleic acid molecule according to the invention and/or with a vector according to the invention, as well as cells, which originate from host cells of this type and which contain the genetic modification according to the invention.

In a preferred embodiment, the invention relates to host cells, in particular prokaryontic or eukaryontic cells, which have been transformed using the nucleic acid molecule according to the invention or a vector according to the invention, as well as host cells, which originate from host cells of this type and which contain the described nucleic acid molecules or vectors according to the invention.
The host cells can be bacteria (e.g. *E. coli*) or fungus cells (e.g. yeast, in particular *S. cerevisiae*, *Agaricus*, in particular *Agaricus bisporus*), as well as vegetable or animal cells. Here, the term "transforms" means that the cells according to the invention are genetically modified with a nucleic acid molecule according to the invention inasmuch as they contain at least one nucleic acid molecule according to the invention in addition to their natural genome. This can be freely present in the cell, possibly as a self-replicating molecule, or it can be stably integrated in the genome of the host cell.
The host cells are preferably microorganisms. Within the framework of the present application, these are understood to mean all bacteria and all protista (e.g. fungi, in particular yeast and algae), as defined, for example, in Schlegel "Allgemeine Mikrobiologie" (Georg Thieme Verlag (1985), 1-2).

It is especially preferred if the host cells according to the invention are plant cells. In principle, these can be plant cells from any plant species, i.e. both monocotyledonous and dicotyledonous plants. Preferably, these will be plant cells

from useful agricultural plants, i.e. from plants, which are cultivated by people for the purposes of food or for technical, in particular industrial purposes. The invention relates preferably to plant cells and plants from starch-storing plants (maize, rice, wheat, rye, oat, barley, cassava, potato, sago, mung bean, pea or sorghum); in particular, plant cells from maize, rice, wheat or potato plants are particularly preferred.

A further subject of the present invention are proteins with the enzymatic activity of a Class 3 branching enzyme, chosen from the group consisting of

a) Proteins, which include the amino acid sequence specified under SEQ ID NO 4;

b) Proteins, which are coded by the coding region of the DNA inserted in the plasmid DSM 15926; or

c) Proteins, which have an identity of at least 70% with the amino acid sequence of the proteins identified under a) or b).

In a further embodiment, the present invention relates to proteins with the enzymatic activity of a Class 3 branching enzyme, wherein the coded protein has an identity of at least 70%, preferably of at least 80%, particularly preferably of at least 90% and especially preferably of 95% with the amino acid sequence specified under SEQ ID NO 4 or with the amino acid sequence of a Class 3 branching enzyme coded by the insertion in plasmid DSM 15926.

In a further embodiment, the invention also relates to proteins, which are coded by nucleic acid molecules according to the invention.

In a preferred embodiment, the present invention relates to a protein with the enzymatic activity of a Class 3 branching enzyme, wherein the Class 3 branching enzyme originates from a potato plant.

Surprisingly, it has been found that plant cells and plants, which have a reduced activity of a Class 3 branching enzyme, synthesise a starch, which is modified in comparison with starch from wild type plant cells or wild type plants.

In conjunction with the present invention, the term "modified starch" means that the starch has changed physical-chemical characteristics compared with non-modified starch obtainable from corresponding wild type plant cells or wild type plants that have not been genetically modified.

In a preferred embodiment of the present invention, the modified starch is native starch.

In conjunction with the present invention, the term "native starch" means that the starch is isolated from plants according to the invention, harvestable plant plants according to the invention or propagation material of plants according to the invention by methods known to the person skilled in the art.

Starch is a classical additive for many foodstuffs in which it essentially takes over the function of binding aqueous additives or increasing the viscosity, or brings about an increased formation of gel. Important characteristic features are the flow and sorption behaviour, the source and sticking temperature, the viscosity and thickening performance, the solubility of the starch, the transparency and paste structure, the heat, shearing and acidic stability, the tendency to retrogradation, the ability to form a film, the freezing/thawing stability, the digestibility as well as the ability to form complexes with, for example, inorganic or organic ions.

In the area of the non-foodstuffs industry, starch can be used, for example, as an auxiliary substance for different manufacturing processes or as an additive in technical products. Particular mention must be made here of the paper and cardboard industry where starch is used as an auxiliary substance. Here, the starch is primarily used for retardation (holding back of solids), the bonding of filler and fine material particles, as a consolidation material and for dehydration. In addition to this, the favourable characteristics of starch with regard to stiffness, hardness, sound, grip, shine, smoothness and resistance to splitting as well as the surfaces are also fully utilised.

A further major area of use of starches is in the adhesive industry, where the possible applications are divided into four sub-areas. Use as a pure starch adhesive, use with starch adhesives prepared with special chemicals, use of starch as an additive to

synthetic resins and polymer dispersions, and the use of starches as a stretching medium for synthetic adhesives.

Furthermore, starches can be used as additives for building materials (e.g. plasterboard sheets, ready-mixed concrete, plaster and mineral fibres), for the manufacture of media for stabilising soil, as a functional aid in plant protection media or fertilisers, as a functional aid in the pharmaceutical industry (e.g. as a bonding medium, tablet dispersal medium, in lubricating and vulnerary powders) and the cosmetic industry (as a carrier of additives), as a strengthening additive for coal and briquettes, as a flocculation medium (e.g. in the preparation of carbon sludge) and as a bonding medium, e.g. in Betonit.

Plant cells according to the invention and plants according to the invention synthesise a modified starch in comparison with starch of corresponding wild type plant cells or wild type plants that have not been genetically modified. In its physical-chemical characteristics, e.g. the amylopectin/amylose ratio, the degree of branching, the phosphate content, the average chain length, the viscosity behaviour, the starch grain size, the side chain distribution and/or the starch grain form, the modified starch is changed in comparison with the synthesised starch in wild type plant cells or plants so that it is better suited for use in particular application areas, for example.

The present invention therefore also relates to modified starches obtainable or isolated from plant cells according to the invention or plants according to the invention, from propagation material according to the invention or from harvestable plant parts according to the invention.

In a particularly preferred embodiment, the present invention relates to modified potato starch.

Furthermore the present invention relates to a method for the manufacture of a modified starch including the step of extracting the starch from a plant cell according to the invention or from a plant according to the invention, from propagation material according to the invention of such a plant and/or from harvestable plant parts according to the invention of such a plant, preferably from starch-storing parts

according to the invention of a plant. Preferably, such a method also includes the step of harvesting the cultivated plants or plant parts and/or the propagation material of these plants before the extraction of the starch and, further, particularly preferably the step of cultivating plants according to the invention before harvesting.

Methods for extracting starches from plants or from starch-storing parts of plants are known to the person skilled in the art. Furthermore, methods for extracting starch from different starch-storing plants are described, e.g. in Starch: Chemistry and Technology (Publisher: Whistler, BeMiller and Paschall (1994), 2nd Edition, Academic Press Inc. London Ltd; ISBN 0-12-746270-8; see e.g. Chapter XII, Page 412-468: Maize and Sorghum Starches: Manufacture; by Watson; Chapter XIII, Page 469-479: Tapioca, Arrowroot and Sago Starches: Manufacture; by Corbishley and Miller; Chapter XIV, Page 479-490: Potato starch: Manufacture and Uses; by Mitch; Chapter XV, Page 491 to 506: Wheat starch: Manufacture, Modification and Uses; by Knight and Oson; and Chapter XVI, Page 507 to 528: Rice starch: Manufacture and Uses; by Rohmer and Klem; Maize starch: Eckhoff et al., Cereal Chem. 73 (1996), 54-57, the extraction of maize starch on an industrial scale is generally achieved by so-called "wet milling".). Devices, which are in common use in methods for extracting starch from plant material are separators, decanters, hydrocyclones, spray dryers and fluid bed dryers.

In conjunction with the present invention, the term "starch-storing parts" is to be understood to mean such parts of a plant in which, in contrast to transitory leaf starch, starch is stored as a deposit for surviving for longer periods. Preferred starch-storing parts are tubers, storage roots, seeds or endosperm; particularly preferred are potato tubers or the endosperm of maize, wheat or rice plants.

Modified starch obtainable by the method according to the invention is also the subject matter of the present invention.

Furthermore, the use of plant cells according to the invention or plants according to the invention for manufacturing a modified starch are the subject matter of the present invention.

The person skilled in the art knows that the characteristics of starch can be changed by thermal, chemical, enzymatic or mechanical derivation, for example. Derived starches are particularly suitable for different applications in the foodstuffs and/or non-foodstuffs sector. The starches according to the invention are better suited as a starting substance for the manufacture of derived starches than conventional starches. In the manufacture of derived starch, they are distinguished by better processing capability and lead to new products, as a modified starch is used as a new starting material for the derivation process.

The present invention therefore also relates to the manufacture of a derived starch, wherein modified starch according to the invention is derived retrospectively.

In conjunction with the present invention, the term "derived starch" is to be understood to mean a modified starch according to the invention, the characteristics of which have been retrospectively changed after isolation from vegetable cells with the help of chemical, enzymatic, thermal or mechanical methods.
In a preferred embodiment of the present invention, the derived starch according to the invention is starch that has been heat-treated and/or acid-treated.
In a further preferred embodiment, the derived starches are starch ethers, in particular starch alkyl ethers, O-allyl ethers, hydroxylalkyl ethers, O-carboxylmethyl ethers, nitrogen-containing starch ethers, phosphate-containing starch ethers or sulphur-containing starch ethers.

In a further preferred embodiment, the derived starches are cross-linked starches.

In a further preferred embodiment, the derived starches are starch graft polymers.

In a further preferred embodiment, the derived starches are oxidised starches.

In a further preferred embodiment, the derived starches are starch esters, in particular starch esters, which have been introduced into the starch using organic

acids. Particularly preferably these are phosphate, nitrate, sulphate, xanthate, acetate or citrate starches.

The derived starches according to the invention are suitable for different applications in the foodstuffs and/or non-foodstuffs sector. Methods for manufacturing derived starches according to the invention are known to the person skilled in the art and are adequately described in the general literature. An overview on the manufacture of derived starches can be found, for example, in Orthoefer (in Corn, Chemistry and Technology, 1987, eds. Watson und Ramstad, Chapter 16, 479-499).

Derived starch obtainable by the method according to the invention for manufacturing a derived starch is also the subject matter of the present invention.

Furthermore, the use of modified starches according to the invention for manufacturing derived starch is the subject matter of the present invention.

Description of sequences

SEQ ID NO 1: Nucleic acid sequence containing the coding region of the 3´-area of a Class 3 branching enzyme from *Solanum tuberosum* (cv Désirée). This sequence is inserted in plasmid AN 46-196.

SEQ ID NO 2: Nucleic acid sequence containing the coding region of the 5´-area of a Class 3 branching enzyme from *Solanum tuberosum* (cv Désirée). This sequence is inserted in plasmid AN 47-196.

SEQ ID NO 3: Nucleic acid sequence containing the full coding region of a Class 3 branching enzyme from *Solanum tuberosum* (cv Désirée). This sequence is inserted in plasmid AN 49 and was deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, 38124 Braunschweig, Germany, in accordance with the Budapest Treaty on 15th September 2003 under the number DSM 15926.

SEQ ID NO 4: Amino acid sequence coding a Class 3 branching enzyme from *Solanum tuberosum* (cv Désirée). This sequence can be derived from the nucleic acid sequence inserted in plasmid AN 49 or from the nucleic acid sequence described under SEQ ID NO 3.

SEQ ID NO 5: Nucleic acid sequence containing the full coding region of a Class 3 branching enzyme from *Solanum tuberosum* (cv Désirée). This sequence has been obtained by combining the nucleic acid sequences described under SEQ ID NO 1 and SEQ ID NO 2. This nucleic acid sequence constitutes an allelic variant of the nucleic acid sequence described under SEQ ID NO 3 coding a Class 3 branching enzyme.

SEQ ID NO 6: Amino acid sequence coding a Class 3 branching enzyme from *Solanum tuberosum* (cv Désirée). This sequence can be derived from the nucleic acid sequence described under SEQ ID NO 5 and constitutes an allelic variant of the amino acid sequence described under SEQ ID NO 4 coding a Class 3 branching enzyme

General methods

The following methods were used in the examples:

Demonstration of the activity of a Class 3 branching enzyme

The activity of a Class 3 branching enzyme was demonstrated with the help of non-denaturing gel electrophoresis as follows:

To isolate proteins from plants, the test material was ground with a pestle in liquid nitrogen, absorbed into an extraction buffer (50 mM Na citrate, pH 6.5; 1 mM EDTA, 4 mM DTT) and, after centrifugation (10 min, 14.000 g, 4 °C), was used directly for measurement of the protein content according to Bradford. Subsequently, 5µg to 20 µg total protein extract was mixed with 4X loading buffer (20% glycerol, 125 mM Tris HCl, pH 6.8) and loaded onto a BE activity gel. The BE activity gel was made up as follows: 2.5 ml 30% acrylamide:0.8% bisacrylamide, 0.1 ml running buffer, 7.4 ml H_2O, 10% ammonium persulphate solution and 5 µl N,N,N',N'-

tetramethylethylenediamine (TEMED). The running buffer (RB) was made up as follows: RB = 30.2 g Tris base, pH 8.0, 144 g glycine on 1 L H_2O. On completion of the gel run, each of the gels was incubated overnight at 37 °C in 25 ml "phosphorylase buffer" (25 ml 1M Na citrate pH 7.0, 0.47 g glucose-1-phosphate, 12.5 mg AMP, 2.5 mg phosphorylase a/b from "rabbit"). The gels were coloured with Lugol's solution.

Starch analysis

a) Determination of the amylose content and of the amylose/amylopectin ratio

Starch was isolated from potato plants by standard methods, and the amylose content and the amylose:amylopectin ratio was determined by the method described by Hovenkamp-Hermelink et al. (Potato Research 31, (1988), 241-246).

b) Determination of the phosphate content

In starch, the positions C2, C3 and C6 of the glucose units can be phosphorylated. To determine the C6-P content of starch, 50 mg of starch are hydrolysed for 4 h at 95°C in 500 µl of 0.7 M HCl. The samples are then centrifuged for 10 minutes at 15500xg and the supernatants are removed. 7 µl of the supernatants are mixed with 193 µl of imidazole buffer (100 mM imidazole, pH 7.4; 5 mM $MgCl_2$, 1 mM EDTA and 0.4 mM NAD). The measurement was carried out in a photometer at 340 nm. After the base absorption had been established, the enzyme reaction was started by addition of 2 units glucose-6-phosphate dehydrogenase (from Leuconostoc mesenteroides, Boehringer Mannheim). The change in absorption is directly proportional to the concentration of the G-6-P content of the starch.

The total phosphate content was determined by the method of Ames (Methods in Enzymology VIII, (1966), 115-118).

Approximately 50 mg of starch are treated with 30 µl of ethanolic magnesium nitrate solution and ashed for 3 hours at 500°C in a muffle oven. The residue is treated with 300 µl of 0.5 M hydrochloric acid and incubated for 30 minutes at 60°C. One aliquot is subsequently made up to 300 µl 0.5 M hydrochloric acid and this is added to a

mixture of 100 µl of 10% ascorbic acid and 600 µl of 0.42% ammonium molybdate in 2 M sulphuric acid and incubated for 20 minutes at 45°C.
This is followed by a photometric determination at 820 nm with a phosphate calibration series as standard.

c) Determination of the viscosity characteristics by means of a Rapid Visco Analyser (RVA)

2 g of starch (DM) are taken up in 25 ml of H_2O (VE-type water, conductivity of at least 15 mega ohm) and used for the analysis in a Rapid Visco Analyser Super3 (Newport Scientific Pty Ltd., Investmet Support Group, Warriewod NSW 2102, Australia). The apparatus is operated following the manufacturer's instructions. The viscosity values are indicated in Centipoise (cP) in accordance with the manufacturer's operating manual, which is incorporated into the description herewith by reference. To determine the viscosity of the aqueous starch solution, the starch suspension is first stirred for 10 seconds at 960 rpm and subsequently heated at 50°C at a stirring speed of 160 rpm, initially for a minute (step 1). The temperature was then raised from 50°C to 95°C at a heating rate of 12°C per minute (step 2). The temperature is held for 2.5 minutes at 95°C (step 3) and then cooled from 95°C to 50°C at 12°C per minute (step 4). In the last step (step 5), the temperature of 50°C is held for 2 minutes. The viscosity is determined during the entire duration.
After the programme has ended, the stirrer is removed and the beaker covered. The gelatinized starch is now available for the texture analysis after 24 hours incubation at room temperature.

The profile of the RVA analysis contains parameters which are shown for the comparison of different measurements and substances. In the context of the present invention, the following terms are to be understood as follows:

1. Maximum viscosity (RVA Max)

The maximum viscosity is understood as meaning the highest viscosity value, measured in cP, obtained in step 2 or 3 of the temperature profile.

2. Minimum viscosity (RVA Min)

The minimum viscosity is understood as meaning the lowest viscosity value, measured in cP, observed in the temperature profile after the maximum viscosity. Normally, this takes place in step 3 of the temperature profile.

3. Final viscosity (RVA Fin)

The final viscosity is understood as meaning the viscosity value, measured in cP, observed at the end of the measurement.

4. Setback (RVA Set)

What is known as the "setback" is calculated by subtracting the value of the final viscosity from that of the minimum occurring after the maximum viscosity in the curve.

5. Gelatinization temperature (RVA PT)

The gelatinization temperature is understood as meaning the point in time of the temperature profile where, for the first time, the viscosity increases drastically for a brief period.

d) Determination of the gel strength (Texture Analyser)

2 g of starch (DM) are gelatinized in the RVA apparatus in 25 ml of an aqueous suspension (temperature programme: see item d) "Determination of the viscosity characteristics by means of a Rapid Visco Analyser (RVA)") and subsequently stored for 24 hours at room temperature in a sealed container. The samples are fixed under the probe (round piston with planar surface) of a Texture Analyser TA-XT2 from Stable Micro Systems (Surrey, UK) and the gel strength was determined using the following parameters:

-	Test speed	0.5 mm/s
-	Depth of penetration	7 mm
-	Contact surface	113 mm^2
-	Pressure	2 g

e) Analysis of the side-chain distribution of the amylopectin by means of ion-exchange chromatography

To separate amylose and amylopectin, 200 mg of starch are dissolved in 50 ml reaction-vessels, using 12 ml of 90% (v/v) DMSO in H_20. After addition of 3 volumes of ethanol, the precipitate is separated by centrifugation for 10 minutes at about

1800xg at room temperature (RT). The pellet is then washed with 30 ml of ethanol, dried and dissolved in 40 ml of 1% (w/v) NaCl solution at 75°C. After the solution has cooled to 30°C, approximately 90 mg of thymol are added slowly, and this solution is incubated for at least 60 h at 30°C. The solution is then centrifuged for 30 minutes at 2000xg (RT). The supernatant is then treated with 3 volumes of ethanol, and the amylopectin which settles out is separated by centrifugation for 5 minutes at 2000xg (RT). The pellet (amylopectin) is then washed with ethanol and dried using acetone. By addition of DMSO to the pellet, one obtains a 1% solution, of which 200 µl are treated with 345 µl of water, 10 µl of 0.5 M sodium acetate (pH 3.5) and 5 µl of isoamylase (dilution 1:10; Megazyme) and incubated for about 16 hours at 37°C. A 1:5 aqueous dilution of this digest is subsequently filtered through a 0.2 µm filter, and 100 µl of the filtrate are analysed by ion chromatography (HPAEC-PAD, Dionex). Separation was performed using a PA-100 column (with suitable precolumn), while detection was performed amperometrically. The elution conditions were as follows:

Solution A - 0.15M NaOH

Solution B – 1 M sodium acetate in 0.15M NaOH

t (min)	Solution A (%)	Solution B (%)
5	0	100
35	30	70
45	32	68
60	100	0
70	100	0
72	0	100
80	0	100
Stop		

Table 1: Composition of the elution buffer for the side chain analysis of the amylopectin at different times during the HPEAC-PAD Dionex analysis. Between the times stated, the composition of the elution buffer changes in each case linearly.

The determination of the relative amount of short side chains in the total of all side chains is carried out via the determination of the percentage of a particular side chain

in the total of all side chains. The total of all side chains is determined via the determination of the total area under the peaks which represent the polymerization degrees of DP 6 to 34 in the HPCL chromatogram.

The percentage of a particular side chain in the total of all side chains is determined via the determination of the ratio of the area under the peak which represents this side chain in the HPLC chromatogram to the total area. The programme Chromelion 6.20 Version 6.20 from Dionex, USA, was used for determining the peak areas.

f) Determination of the activity of the BEIII protein

This was carried out as specified in the example.

g) DSC-analysis („Differential Scanning Calorimetry")

Investigations with the aid of DSC-analysis have been done by the method described by WO 01/19975. 10 mg starch treated with 30 µl H_20 (VE-type water, conductivity of at least 15 mega ohm) were sealed in stainless steal pans (volume 50 µl). The pan is heated from 20°C to 150°C at a rate of 10°C per minute in a Diamond DSC-instrument (Perkin Elmer). The programme Pyres from Perkin Elmer was used for determining the data.

Examples

Example 1

Cloning of a full-length sequence coding a Class 3 branching enzyme from Solanum tuberosum

The gene sequence coding for this Class 3 branching enzyme in *Solanum tuberosum* has not previously been described .

By sequence comparisons with different branching enzymes, a domain was identified, with the help of which EST databases were examined. In doing so, the EST TC73137 (TIGR database; http://www.tigr.org/tigr-scripts/tgi/tc_report.pl?tc=TC73137&species=potato) from potato was identified.

With the help of the primers B1_Asp (GAT GGG TAC CAG CAC TTC TAC TTG GCA GAG G) and B2_Sal (TCA AGT CGA CCA CAA CCA GTC CAT TTC TGG), a sequence from a tuber-specific cDNA bank from *Solanum tuberosum* (cv. Désirée) corresponding to this EST sequence was amplified. Attempts to use leaf-specific, "sink"-tissue-specific or "source"-tissue-specific cDNA banks as a template for the PCR reaction led to no amplification.

In order to amplify the whole coding sequence of the branching enzyme concerned, which up to now had also included unknown sequences, primers were manufactured, which were complimentary to the ends of the previously known sequence and vector sequences of the cDNA banks concerned. With all the primer combinations for the amplification of a full-length sequence of a Class 3 branching enzyme used in this approach, it was not possible to amplify any further area. Hereupon, EST databases of tomato were examined again.

In this case, two ESTs from tomato were identified (TIGR database; BG127920 and TC130382), which either had a high homology to the amplification of the Class 3 branching enzyme from potato described above (TC130382) and (BG127920) respectively, or to the putative branching enzyme gene from arabidopsis (GenBank: GP|9294564|dbj|BAB02827.1).

Primers were now manufactured again in order to also amplify previously unknown sequences of the Class 3 branching enzyme. By means of PCR, the 3'-area of the Class 3 branching enzyme was amplified from a cDNA bank, made from tubers of *Solanum tuberosum* (cv. Désirée), with the primers KM2_Spe (5'-TCAAACTAGTCACAACCAGTCCATTTCTGG-3') and So_putE (5'-CACTTTAGAAGGTATCAGAGC-3'). The fragment with a size of ca. 1 kb that was obtained was cloned undirectedly in the pCR4-TOPO vector from Invitrogen (product number: 45-0030). The plasmid produced was designated as AN 46-196. The sequence of the inserted fragments in the plasmid AN 46-196 is shown under SEQ ID NO 1.

The 5'-area was likewise amplified by means of PCR technology and using the primers So_put5' (5'-GTATTTCTGCGAAGGAACGACC-3') and So_putA (5'-AACAATGCTCTCTCTGTCGG-3') from the same cDNA bank. The fragment with a

size of ca. 2 kb that was obtained was cloned undirectedly in the pCR4-TOPO vector from Invitrogen (product number: 45-0030). The plasmid produced was designated as AN 47-196. The sequence of the inserted fragments in the plasmid AN 47-196 is shown under SEQ ID NO 2.

Primers were now manufactured again in order to amplify a full-length sequence. The following primers were used: SO_putA (AACAATGCTCTCTCTGTCGG) and SO_putE (CACTTTAGAAGGTATCAGAGC). A PCR product with an approximate size of 3.2 kb was obtained and was cloned in the pCR2.1 vector from Invitrogen (product number: 45-0030). The plasmid obtained (filed under DSM 15926) was designated as AN 49. The sequence of the inserted fragments in the plasmid AN 49 is shown under SEQ ID NO 3.

Example 2

Information on vectors and plasmids

Information on vector AN 54-196

AN 54-196 is a derivative of the plasmid pBinB33-Hyg, to which was added a part sequence of the Class 3 branching enzyme gene as an "inverted repeat, (RNAi technology) under the control of the promoters of the patatin gene B33 from *Solanum tuberosum* (Rocha-Sosa et al., 1989). For this purpose, first of all, a PCR product with the primers B1_Asp (GAT GGG TAC CAG CAC TTC TAC TTG GCA GAG G) and B2_Sal (TCA AGT CGA CCA CAA CCA GTC CAT TTC TGG) from a tuber-specific cDNA bank from *Solanum tuberosum* (cv. Désirée) was amplified, as a result of which the sites Asp718 and SalI were added. The PCR product obtained (625 bp) was cioned in "antisense" orientation to the B33 promoter via these two sites. A second PCR fragment, which was amplified with the primers B3_Sal (GCT TGT CGA CGG GAG AAT TTT GTC CAG AGG) and B4_Sal (GAT CGT CGA CAG CAC TTC TAC TTG GCA GAG G) from a tuber-specific cDNA bank from *Solanum tuberosum* (cv. Désirée) and which is identical to the 301 bp of the first fragment, was cloned via

the SalI site behind the first fragment, but in "sense" orientation to the B33 promoter. This arrangement is described as "inverted repeat" (RNAi technology).

Information on vector pBinB33-Hyg

Starting from the plasmid pBinB33, the *Eco*RI-*Hind*III fragment including the B33 promoter, a part of the polylinker, and the *ocs* terminator were cut out and spliced into the correspondingly cut vector pBIB-Hyg (Becker, 1990).

The plasmid pBinB33 was obtained by splicing the promoter of the patatin gene B33 from *Solanum tuberosum* (Rocha-Sosa et al., 1989) as a *Dra*I fragment (nucleotide – 1512 - +14) into the vector pUC19 cut with *Sst*I, the ends of which had been smoothed with the help of the T4 DNA polymerase. This resulted in the plasmid pUC19-B33. The B33 promoter was cut out from this plasmid with *Eco*RI and *Sma*I and spliced into the correspondingly cut vector pBinAR. This resulted in the vegetable expression vector pBinB33.

The plasmid pBinAR is a derivative of the vector plasmid pBin19 (Bevan, 1984) and was constructed as follows:

A fragment of length 529 Bp, which included the nucleotides 6909-7437 of the 35S RNA promoter of the cauliflower mosaic virus (Pietrzak et al., 1986, Nucleic Acids Research 14, 5857-5868), was isolated as an *Eco*RI/*Kpn*I fragment from the plasmid pDH51 (Pietrzak et al., 1986) and spliced between the *Eco*RI and *Kpn*I sites of the polylinker from pUC18. This resulted in the plasmid pUC18-35S.

With the help of the restriction endonucleases HindIII und PvuII, a fragment of length 192 Bp, which included the polyadenylation signal (3'-end) of the octopin synthase gene (gene 3) of the T-DNA of the Ti plasmid pTiACH5 (Gielen et al., 1984) (nucleotides 11749-11939) was isolated from the plasmid pAGV40 (Herrera-Estrella et al., 1983). After the addition of *Ssp*I linkers to the *Pvu*II site, the fragment was spliced between the *Sph*I and *Hind*III site from pUC18-35S. This resulted in the plasmid pA7.

The whole polylinker containing the 35S promoter and the ocs terminator with EcoRI and HindIII was cut out of pA7 and spliced into the correspondingly cut pBin19. This resulted in the vegetable expression vector pBinAR (Höfgen and Willmitzer, 1990).

Example 3

Genetically modified plants with reduced Class 3 branching enzyme activity

In order to produce transgenic potato plants, which have a reduced expression of a Class 3 branching enzyme gene, the T-DNA of the plasmid AN 54-196 was transferred into potato plants of the variety Désirée with the help of agrobacteria, as described in Rocha-Sosa et al. (EMBO J. 8, (1989), 23-29). The plants of the variety Désirée obtained by transformation with the plasmid AN 53-196 were designated as 369SO.

Analysis with the help of non-denaturising gel electrophoresis of protein extracts from tubers of wild type plant cells and/or protein extracts from genetically modified plants (396SO), showed that the genetically modified plant cells have a reduced activity of a Class 3 branching enzyme in comparison with protein extracts from tubers of wild type plant cells.

Additionally mRNA of tuber material was extracted with standard methods and applied to quantitative RT-PCR analysis. The analysis were performed with a PCR-instrument ABI Prism 7700 form Applied Biosystems using the primer St_BE-f2 (5'-TCA GGT CTA CAA GTT GAC CCG A-3'), St_BE-r2 (5'-GTA GAA CCT TCC CTT TTG TGT GA-3') and St_BE-Fam (5'-Fam-CAT GAT CAC TCT AGC AAT CAA AGT GCC-Tamra-3'). It could be shown that given plants showed reduced transcript in comparison with the corresponding wild type.

Example 4

Potato starch extraction process

All tubers of one line (0,3 to 0,7 kg) are processed jointly in a commercially available juice extractor (Multipress automatic MP80, Braun). The starch-containing fruit water is collected in a 1-l bucket (ratio bucket height: bucket diameter = approx. 1.1) containing 20 ml of tap water together with a spoon-tipful (approx. 0,3-0,4 g) of

sodium disulphite. The bucket is subsequently filled completely with tap water. After the starch has been allowed to settle for 2 hours (h), the supernatant is decanted off, the starch is resuspended in 1 l of tap water and poured over a sieve with a mesh size of 125 µm. After 2 h (starch has again settled at the bottom of the bucket), the aqueous supernatant is again decanted off. This wash step is repeated 3 more times so that the starch is resuspended a total of 5 times in fresh tap water. Thereafter, the starches are dried at 37°C to a water content of 12-17% and homogenized using a pestle and mortar. The starches are now available for analyses.

Example 5

Analysis of the starch from plants with reduced BEIII gene expression

The starch from various independent lines of plants named 369SO were isolated from potato tubers. The physico-chemical properties of this starch were subsequently analysed. The results of the characterization of the modified starches are shown in the following for an example of a selection of certain plant lines. The analyses were carried out by the methods described hereinabove.

a) RVA Analysis

	RVA Max (%)	RVA Min (%)	RVA Fin (%)	RVA Set (%)	RVA PT (%)	Gel strength
cv.Desiree	100	100	100	100	100	100
369SO048	91	64	90	N.d.	98	128
369SO050	84	84	89	112	98	127
369SO052	94	85	88	101	98	N.d.
369SO106	91	87	89	99	98	N.d.
369SO129	87	88	93	114	99	138

Table 2: Parameters of the RVA analysis of starch isolated from wild-type plants (cv. Desiree), plants with a reduced activity of a BEIII protein (369SO) in per cent based on data of starch of the wild type. The RVA analysis was carried out as described in general methods. N.d. = not determined.

b) Analysis of the phosphate and Amylose content

No.	Genotype	Phosphate in C6 (%)	Total phosphate in (%)	Amylose (%)	Amylose (% WT)
1	cv. Desiree	100,0	100,0	21,3	100,0
2	369SO048	72,8	84,8	20,8	97,7
3	369SO050	79,2	78,0	20,1	94,4
4	369SO052	84,8	83,2	19,6	92,0
5	369SO106	84,8	85,9	20,1	94,4
6	369SO129	80,8	81,2	20,2	94,8

Table 3: Phosphate and amylose contents of starch isolated from wild-type plants (cv. Desiree), plants with a reduced activity of BEIII protein (369SO). The phosphate contents in the C6 position of the glucose monomers and the total phosphate content of the starch are indicated in per cent based on starch from wild-type plants; amylose contents are indicated in per cent amylose based on the total amount of the starch, or in per cent based on the amylose content of starch from wild-type plants.

c) Analysis of side-chain distribution

The analysis of the side-chain distribution of the amylopectin was carried out as described above. The table which follows is a summary of the contributions of the individual peak areas:

Glucose units	cv. Desiree	369SO 048	369SO 050	369SO 052	369SO 106	369SO 129
dp 6	2,19	2,57	2,83	2,78	2,59	2,59
dp 7	1,69	1,76	1,84	1,85	1,85	1,73
dp 8	1,35	1,34	1,37	1,38	1,44	1,36
dp 9	2,26	2,27	2,31	2,32	2,42	2,31
dp 10	3,74	3,81	3,86	3,94	4,00	3,85
dp 11	5,13	5,23	5,30	5,45	5,37	5,30
dp 12	5,99	6,14	6,18	6,32	6,17	6,17
dp 13	6,40	6,53	6,54	6,63	6,48	6,63
dp 14	6,39	6,45	6,44	6,49	6,37	6,52
dp 15	6,11	6,14	6,12	6,15	6,05	6,09
dp 16	5,74	5,75	5,72	5,75	5,68	5,72
dp 17	5,37	5,35	5,35	5,35	5,30	5,35
dp 18	5,08	5,04	5,06	5,05	5,01	5,06
dp 19	4,89	4,86	4,88	4,84	4,83	4,86
dp 20	4,68	4,59	4,65	4,60	4,60	4,62

Glucose units	cv. Desiree	369SO 048	369SO 050	369SO 052	369SO 106	369SO 129
dp 6	100	117,4	129,2	126,9	118,3	118,3
dp 7	100	104,1	108,9	109,5	109,5	102,4
dp 8	100	99,3	101,5	102,2	106,7	100,7
dp 9	100	100,7	102,4	102,9	107,3	102,4
dp 10	100	102,0	103,3	105,5	107,1	103,1
dp 11	100	102,0	103,4	106,3	104,8	103,4
dp 12	100	102,5	103,2	105,5	103,0	103,0
dp 13	100	102,1	102,3	103,7	101,3	103,7
dp 14	100	100,9	100,8	101,6	99,7	102,0
dp 15	100	100,5	100,2	100,7	99,0	99,7
dp 16	100	100,3	99,7	100,3	99,0	99,7
dp 17	100	99,7	99,7	99,7	98,8	99,7
dp 18	100	99,3	99,7	99,5	98,7	99,7
dp 19	100	99,5	99,9	99,1	98,9	99,5
dp 20	100	98,1	99,4	98,3	98,3	98,7
dp 21	100	99,5	99,1	98,0	98,4	98,6
dp 22	100	98,8	99,0	97,6	98,0	98,0
dp 23	100	98,8	97,7	96,2	98,3	98,8
dp 24	100	98,4	96,9	96,0	98,1	96,9
dp 25	100	96,9	95,9	94,0	96,6	97,2
dp 26	100	97,1	94,2	93,5	96,4	95,6
dp 27	100	95,8	93,8	91,7	95,0	95,0
dp 28	100	96,1	92,3	91,3	95,2	93,7
dp 29	100	97,2	92,0	90,3	95,5	94,9
dp 30	100	94,0	91,4	89,4	93,4	93,4
dp 31	100	95,6	90,8	89,2	92,4	94,0
dp 32	100	94,2	89,3	89,3	92,2	92,2
dp 33	100	92,2	89,8	91,0	93,4	93,4
dp 34	100	91,9	88,9	90,4	93,3	91,9

Table 5: The table shows a summary of the contributions of the individual peak areas of the HPAEC chromatogram in per cent based on starch from wild-type plants.

d) Analysis of the amylopectin side chain distribution by means of gel permeation chromatography

Analysis of the amylopectin side chain distribution by means of gel permeation chromatography were additionally performed.

To separate amylose and amylopectin, 100 mg of starch are dissolved in 6 ml of 90% strength (v/v) DMSO with constant stirring. After addition of 3 volumes of ethanol, the precipitate is separated off by centrifugation for 10 minutes at 1800xg at room temperature. The pellet is subsequently washed with 30 ml of ethanol, dried and dissolved in 10 ml of 1% strength (w/v) NaCl solution at 60°C. After cooling the

solution to 30°C, approximately 50 mg of thymol are added slowly, and this solution is incubated for 2 to 3 days at 30°C. The solution is subsequently centrifuged for 30 minutes at 2000xg at room temperature. The supernatant is treated with three volumes of ethanol, and the amylopectin which precipitates is separated off by centrifugation for 5 minutes at 2000xg at room temperature. The pellet (amylopectin) is washed with 10 ml of 70% strength (v/v) ethanol, centrifuged for 10 minutes at 2000xg at room temperature and then dried using acetone.

10 mg of amylopectin are subsequently stirred for 10 minutes at 70°C in 250 µl of 90% strength (v/v) DMSO. 375 µl of water at a temperature of 80°C are added to the solution until dissolution is complete.

200 µl of this solution are treated with 300 µl of a 16.6 mM sodium acetate solution pH 3.5 and 2 µl of isoamylase (0.24 u/µl, Megazyme, Sydney, Australia) and the mixture is incubated for 15 hours at 37°C.

A 1:4 dilution of this aqueous isoamylase reaction mixture with DMSO, comprising 90 mM sodium nitrate, is subsequently filtered through a 0.2 µm filter, and 24 µl of the filtrate is analysed chromatographically. Separation was carried out with two columns connected in series, first a Gram PSS3000 (Polymer Standards Service, with suitable precolumn), followed by a Gram PSS100. Detection was by means of refraction index detector (RI 71, Shodex). The column was equilibrated with DMSO comprising 90 mM sodium nitrate. It was eluted with DMSO comprising 90 mM sodium nitrate at a flow rate of 0.7 ml/min over a period of 1 hour.

To correlate the elution volume with the molecular mass, the column used was calibrated with dextran standards. The dextrans used, their molecular mass and the elution volumes are shown in Table 6. Using the resulting calibration graph, the elution diagram was pictured as a molecular weight distribution.

The chromatograms obtained were further evaluated using the program Wingpc Version 6 from Polymer Standards Service GmbH, Mainz, Germany.

The total area under the line of the GPC chromatogram was divided into individual segments, each of which represent groups of side chains of different lengths. The chosen segments contained glucan chains with the following degree of polymerization (DP = number of glucose monomers within one side chain): DP<12, DP12-18, DP19-24, DP25-30, DP31-36, DP37-42, DP43-48, DP49-55, DP56-61 and DP62-123. To determine the molecular weight of the individual side chains, a

molecular weight of 162 was assumed for glucose. The total area under the line in the GPC chromatogram was then set as 100%, and the percentage of the areas of the individual segments was calculated based on the percentage of the total area. Results obtained from this analysis are shown in Table 7.

elution volume [ml]	molar mass [D]	sample
18,76	401300	Dextran T670
19,41	276500	Dextran T410
20,49	196300	Dextran T270
21,35	123600	Dextran T150
22,45	66700	Dextran T80
23,52	43500	Dextran T50
25,15	21400	Dextran T25
26,92	9890	Dextran T12
28,38	4440	Dextran T5
30,77	1080	Dextran T1

Table 6: Calibration table.

degree of	% total area					
polymerisation	cv.Desiree	369 SO 48	369 SO 50	369 SO 52	369 SO 106	369 SO 129
<dp12	16,49	16,57	17,07	17,73	17,59	17,64
dp12-19	13,89	14,47	14,22	14,82	14,16	14,23
dp20-25	15,74	16,54	16,15	16,74	16,32	16,51
dp26-31	9,41	9,73	9,57	9,80	9,86	9,88
dp32-37	8,53	8,53	8,45	8,56	8,59	8,45
dp38-43	6,82	6,67	6,57	6,63	6,58	6,44
dp44-49	6,05	5,91	5,81	5,83	5,79	5,72
dp50-56	4,88	4,78	4,71	4,66	4,70	4,67
dp57-62	4,26	4,15	4,10	3,98	4,09	4,10
dp63-123	13,92	12,66	13,35	11,25	12,31	12,38

Table 7: Side chain profiles DP<12, DP 12 to 18, DP 19 to 24, DP 25 to 30, DP 31 to 36, DP 37 to 42, DP 43-48, DP 49 to 55, DP 56 to 61 and DP 62 to 123 for amylopectin isolated from wild-type plants (cv. Desiree) and from plants with a reduced activity of a BEIII protein (369SO).

degree of	% WT					
polymerisation	cv.Desiree	369 SO 48	369 SO 50	369 SO 52	369 SO 106	369 SO 129
<dp12	100,00	100,47	103,53	107,53	106,69	106,96
dp12-19	100,00	104,13	102,37	106,68	101,90	102,40
dp20-25	100,00	105,11	102,62	106,32	103,69	104,87
dp26-31	100,00	103,34	101,67	104,09	104,81	104,92
dp32-37	100,00	100,01	99,05	100,38	100,73	99,04
dp38-43	100,00	97,74	96,40	97,23	96,54	94,48
dp44-49	100,00	97,68	96,00	96,27	95,76	94,48
dp50-56	100,00	97,90	96,45	95,57	96,26	95,78
dp57-62	100,00	97,36	96,07	93,44	95,91	96,13
dp63-123	100,00	90,95	95,89	80,82	88,41	88,89

Table 8: Side chain profiles DP<12, DP 12 to 18, DP 19 to 24, DP 25 to 30, DP 31 to 36, DP 37 to 42, DP 43-48, DP 49 to 55, DP 56 to 61 and DP 62 to 123 for

amylopectin isolated from wild-type plants (cv. Desiree) and from plants with a reduced activity of a BEIII protein (369SO). The percentages indicate the modification of the individual side chain profiles based on amylopectin isolated from wild-type plants.

e) DSC-analysis („Differential Scanning Calorimetry")

Investigations with the aid of DSC-analysis („Differential Scanning Calorimetry") have been done by the method described by WO 01/19975. Results obtained from this analysis are shown in Table 9.

	T0 (°C)	T0 (%)	T Peak (°C)	T Peak (%)	dH (J/g)	dH (J/g)
cv.Desiree	64,84	100	68,09	100	20,31	100
369SO048	64,32	99,2	67,16	98,6	20,33	100,1
369SO050	63,35	97,7	66,75	98,0	20,63	101,6
369SO052	63,27	97,6	66,46	97,6	21,23	104,5
369SO106	63,77	98,3	66,96	98,3	21,42	105,5
369SO129	63,75	98,3	67,41	99,0	20,57	101,3

Table 9: Parameters of the DSC analysis of starch isolated from wild-type plants (cv. Desiree), plants with a reduced activity of a BEIII protein (369SO) indicated in °C respectively J/g and in per cent based on data of starch of the wild type. The DSC analysis was carried out as described in general methods. T0 [°C] = peak onset, T Peak [°C] = Peak temperature, dH [J/g] = heat of melting.